### Polyphenols

Potentiation of the bioavailability of blueberry phenolic compounds by co-ingested grape phenolic compounds in mice, revealed by targeted metabolomic profiling in plasma and feces. Food Funct. 2016.

Dietary polyphenol supplementation prevents alterations of spatial navigation in middle-aged mice. Front Behav Neurosci. 2016.

Short-term supplementation with a specific combination of dietary polyphenols increases energy expenditure and alters substrate metabolism in overweight subjects. Int J Obes. 2014.


Acute effects of polyphenols from cranberries and grape seeds on endothelial function and performance in elite athletes: Randomized, placebo-controlled, cross-over studies. Sports. 2013.


### Polyunsaturated Fatty Acids


Effect of DHA-rich fish oil on PPARγ target genes related to lipid metabolism in type 2 diabetes: A randomized, double-blind, placebo-controlled clinical trial. J Clin Lipidol. 2015


### Proteolytic Enzymes


Proteolytic enzyme combination reduces inflammation and oxidative stress and improves insulin sensitivity in a model of metabolic syndrome. Adv Enzyme Res. 2015.


### Vitamins and Minerals


A randomized trial of nutrient supplements to minimise psychiatric illness after a natural disaster. Psych Res. 2015.


Hip bone loss is attenuated with 1000 IU but not 400 IU daily vitamin D3: a 1 year double-blind RCT in postmenopausal women. J Bone Min Res. 2013.

Effect of selenium supplementation on CD4+ T-cell recovery, viral suppression and morbidity of HIV-infected patients in Rwanda: a randomized controlled trial. AIDS. 2015.

Effect of selenium supplementation on CD4 T-cell recovery, viral suppression, morbidity and quality of life of HIV-infected patients in Rwanda: study protocol for a randomized controlled trial. Trials. 2011.
Antioxidants


Probiotics


Botanicals


Phase II trial of encapsulated ginger as a treatment for chemotherapy-induced nausea and vomiting. Support Care Cancer. 2009.


Other


INDEX

α-lipoic acid (24)
acetyl-l-carnitine (24)
aging (2, 11, 21)
antioxidants (24)
athletes (5, 6)
behavior (29)
blueberry (1, 2, 7)
bone health (21)
brain (2, 11, 29)
cardiovascular health (5, 9, 20, 25)
children (18)
cranberry (4, 5, 7)
cognitive function (2, 11)
DHA (9, 10, 11, 12)
endothelial function (5)
enzymes (13, 14, 15, 16, 17)
EPA (9, 11, 12)
fish oil (9, 10, 11, 12)
ginger (26, 27, 28)
grape (1, 2, 5)
healthy subjects (5, 6, 12, 28)
immune (22, 23)
inflammation (6, 7, 8, 9, 13, 14)
joints (13, 17)
metabolic health (3, 10, 25)
multivitamin (19, 23)
mineral (22, 23)
mood (19, 24)
nausea (27)
neurotransmission (7, 29)
oignon (4)
overweight subjects (3, 9, 10)
performance (5, 6)
polyphenols (1, 2, 3, 4, 5, 6, 7, 8)
pregnenolone (29)
probiotics (25)
proteases (13, 14, 15, 16, 17)
quercetin (4)
resveratrol (3, 6, 8)
selenium (22, 23)
spinach (7)
strawberry (4, 7)
sports (5, 6)
Vitamin B (19)
Vitamin D (18, 21)
POLYPHENOLS

Potentiation of the bioavailability of blueberry phenolic compounds by co-ingested grape phenolic compounds in mice, revealed by targeted metabolomic profiling in plasma and feces. Food Funct. 2016.

Dietary polyphenol supplementation prevents alterations of spatial navigation in middle-aged mice. Front Behav Neurosci. 2016.

Short-term supplementation with a specific combination of dietary polyphenols increases energy expenditure and alters substrate metabolism in overweight subjects. Int J Obes. 2014.


Acute effects of polyphenols from cranberries and grape seeds on endothelial function and performance in elite athletes: Randomized, placebo-controlled, cross-over studies. Sports. 2013.


Potentiation of the bioavailability of blueberry phenolic compounds by co-ingested grape phenolic compounds in mice, revealed by targeted metabolomic profiling in plasma and feces.

Food Funct. 2016.
Potentiation of the bioavailability of blueberry phenolic compounds by co-ingested grape phenolic compounds in mice, revealed by targeted metabolomic profiling in plasma and feces†

Stéphanie Dudonné,⁎a Alexandre Dal-Pan,a,b Pascal Dubé,a Thibault V. Varin,a Frédéric Calon,a,b and Yves Desjardinsa

The low bioavailability of dietary phenolic compounds, resulting from poor absorption and high rates of metabolism and excretion, is a concern as it can limit their potential beneficial effects on health. Targeted metabolomic profiling in plasma and feces of mice supplemented for 15 days with a blueberry extract, a grape extract or their combination revealed significantly increased plasma concentrations (3–5 fold) of blueberry phenolic metabolites in the presence of a co-ingested grape extract, associated with an equivalent decrease in their appearance in feces. Additionally, the repeated daily administration of the blueberry–grape combination significantly increased plasma phenolic concentrations (2–3-fold) compared to animals receiving only a single acute dose, with no such increase being observed with individual extracts. These findings highlight a positive interaction between blueberry and grape constituents, in which the grape extract enhanced the absorption of blueberry phenolic compounds. This study provides for the first time in vivo evidence of such an interaction occurring between co-ingested phenolic compounds from fruit extracts leading to their improved bioavailability.

1. Introduction

In contrast to drugs, which have a targeted and well-defined molecular mechanism of action, phytochemicals act in a pleiotropic manner.1−4 The multiple protective effects of plant bioactives on health are therefore attributed to a combined effect of several interacting compounds, rather than to a single molecule.5,6 Positive interactions (synergism) between phytochemicals can enhance the potency of a bioactive compound, thereby leading to a combined bioactivity greater than the sum of the individual compounds, while negative interactions (antagonism) result in a reduced bioactivity from what is expected.2 Synergies between plant bioactives have been widely suggested,7−9 but few reports have demonstrated this phenomenon or proposed the possible underlying mechanisms. In particular, interactions between phytochemicals have been suggested to affect the way they are absorbed, metabolized and excreted,10,11 but to date, in vivo evidence of an improvement of their bioavailability resulting from such interactions is lacking, owing to methodological limitations. Due to the tremendous diversity of phytochemicals, their variable absorption and their complex biotransformation, it has been almost impossible to comprehensively assess their bioavailability by conventional methods. Metabolomics now provides suitable approaches to analyze changes in metabolite profiles related to synergistic or antagonistic effects, and may thereby improve our understanding of the complex interactions inherent to multi-target and multi-component phytotherapeutics.12

Berries are rich sources of phenolic compounds and are receiving growing interest, because of their positive effects on health.13 Epidemiological, pre-clinical and cell culture studies support the use of phenolic compounds to prevent chronic diseases, such as cardiovascular diseases and certain types of cancers.14,15 In particular, blueberry and grape are being studied for their capacity to prevent neurodegeneration and cognitive decline.16−18 The purpose of the present work was to investigate the bioavailability of phenolic compounds from blueberry and grape extracts after oral administration in mice, using targeted metabolomics in plasma and feces, and determine whether acute or chronic co-administration might affect their bioavailability.
2. Materials and methods

2.1. Plant material, chemicals and phenolic characterization

The Neurophenols Consortium extract is a standardized phenol-rich combination of blueberry (Vaccinium angustifolium Ait.) and grape (Vitis vinifera L.) extracts. This blend and the individual extracts were provided by Nutra Canada (Canada) and Activ'Inside (France). Phenolic standards and reagents were purchased from Sigma-Aldrich (St Louis, MO, USA), with the exception of malvidin 3-glucoside, which was purchased from Extrasynthese (Genay, France). Liquid chromatography grade solvents and acids were respectively purchased from EMD Millipore Chemicals (Billerica, MA, USA) and Anachemia (Montreal, QC, Canada). Fruit extracts were characterized using a previously described methodology. Briefly, total phenolic content was determined using the Folin–Ciocalteu reagent and quantified as gallic acid equivalent. Anthocyanins were analyzed by reverse-phase HPLC with DAD detection and quantified using malvidin 3-glucoside standard. Proanthocyanidins were separated according to their degree of polymerization (DP) by normal-phase HPLC, and quantified by fluorescence detection using the (−)-epicatechin standard. Flavonols, phenolic acids and resveratrol were analyzed using reverse-phase UHPLC coupled to tandem mass spectrometry. Phenolic compounds were quantified using their high-purity commercial standards when available, aglycone or most similar phenolic structures otherwise.

2.2. Animal study, treatments and sample collection

Seventy-two 4 month-old male C57Bl/6j mice (Jackson laboratory, Bar Harbor, ME, USA) were individually maintained in cages in temperature- and humidity-controlled rooms (21 ± 2°C, 35–40%) with a daily 12 h–12 h light–dark cycle, and fed a control diet free of phenolic compounds (Teklad 2018, Harlan, KY, USA). Animal facilities and procedures met the guidelines of the Canadian Council on Animal Care, and the protocols were approved by the Animal Care Committee of Laval University (protocol 13-114). Animals were divided into 2 groups to perform separate acute and chronic studies (see experimental design in Fig. 1). For each study, animals were randomly assigned to 4 different groups. The animals of 3 treated groups (n = 10) were supplemented either with a blueberry extract (B), a grape extract (G) or their combination (BG), while the animals of a control group (C, n = 6) received only the vehicle (water), all administered by intragastric gavage in a final volume of 150 μl. BG provided a phenol intake of 297.5 mg per kg body weight (BW), while B and G were individually administered at their exact proportion in the BG blend, leading to phenol intakes of 31.1 and 266.4 mg kg⁻¹ respectively. Phenolic contributions of each treatment are detailed in Table 1.

2.2.1. Acute study. After 7 days of acclimation, all the animals were fasted for 2 hours in order to collect baseline blood samples (D0t0, Fig. 1). Thirty minutes following oral gavage with their respective treatment (D0t30), the animals were sacrificed under deep anesthesia with ketamine/xylazine. Intracardiac blood samples were collected using EDTA-containing syringes and immediately centrifuged (3000 rpm, 5 min at 4°C) in order to separate plasma.

2.2.2. Chronic study. After 7 days of acclimation, all the animals were fasted for 2 hours in order to collect baseline blood samples (D0t0, Fig. 1). The animals then received their respective treatments at the same time each day, over the course of 15 days. On the last day of the study, blood samples were collected prior to the last supplementation (D15t0) and before and 30 minutes after the last gavage in the chronic study (D15t30 and D15t30 respectively). Feces were collected in the chronic study before (D0) and throughout the 15-day supplementation (D1–D15).

Fig. 1 Experimental design. Mice were administered a single dose (acute study) or a daily dose for 15 days (chronic study) of blueberry (B), grape (G) or blueberry–grape (BG) extracts, or water (control group, C), by oral gavage. Plasma samples were collected before the supplementation in each study (D0t0), 30 minutes after the single oral gavage in the acute study (D0t30), and before and 30 minutes after the last gavage in the chronic study (D15t0 and D15t30 respectively). Feces were collected in the chronic study before (D0) and throughout the 15-day supplementation (D1–D15).
Phase (µSPE) as previously described,19 with slight modifications. The homogenate was centrifuged twice at 15,000 rpm at 4 °C for 15 min. Phenolic metabolites were disrupted phenol groups during the 15 days of supplementation.

This journal is © The Royal Society of Chemistry 2016

Food Funct.

Table 1: Intake of phenolic compounds in mice

<table>
<thead>
<tr>
<th>Phenolic compounds</th>
<th>Intake (mg per kg BW)</th>
<th>Dose (mg per kg BW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>G</td>
</tr>
<tr>
<td>Flavan-3-ols/proanthocyanidins</td>
<td>1.5</td>
<td>204.0</td>
</tr>
<tr>
<td>DP 1–3</td>
<td>0.6</td>
<td>170.4</td>
</tr>
<tr>
<td>(−)-Catechin + (−)-epicatechin</td>
<td>&lt;0.1</td>
<td>128.7</td>
</tr>
<tr>
<td>DP &gt;3</td>
<td>0.9</td>
<td>33.6</td>
</tr>
<tr>
<td>Anthocyanins</td>
<td>2.2</td>
<td>—</td>
</tr>
<tr>
<td>Malvidin + glycosides</td>
<td>1.1</td>
<td>—</td>
</tr>
<tr>
<td>Others</td>
<td>1.1</td>
<td>—</td>
</tr>
<tr>
<td>Phenolic acids</td>
<td>13.5</td>
<td>4.7</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>9.8</td>
<td>—</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.6</td>
<td>4.3</td>
</tr>
<tr>
<td>Others</td>
<td>3.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Flavanols</td>
<td>4.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Quercetin + glycosides</td>
<td>4.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Others</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Stilbenes (resveratrol)</td>
<td>—</td>
<td>0.2</td>
</tr>
<tr>
<td>Total</td>
<td>31.1</td>
<td>266.4</td>
</tr>
</tbody>
</table>

"Total phenolic intake was determined using the Folin–Ciocalteu assay. BW: body weight, DP: polymerization degree. B: blueberry extract, G: grape extract, BG: blueberry-grape extract.

Table 2. Intake of phenolic compounds in mice

During the whole supplementation period (D0 and D1–15, respectively) and were freeze-dried. Plasma and feces samples were maintained at −80 °C until analysis. No variations in body weight or food consumption were observed between the groups during the 15 days of supplementation.

2.3. Extraction and characterization of phenolic metabolites from plasma and feces

Plasma and dried feces were mixed with 4% phosphoric acid (v/v and with a material–solvent ratio of 1:4 respectively) to disrupt phenol–protein binding. Feces were ground with glass beads using a Biospec BeadBeater (Bartlesville, OK, USA) for 15 seconds and the homogenate was centrifuged twice at 15,000 rpm at 4 °C for 15 min. Phenolic metabolites were characterized by UHPLC-MS/MS after micro-extraction on solid phase (µSPE) as previously described,19 with slight modifications. Acidified plasma or feces supernatants were loaded into preconditioned Waters OASIS HLB micro-elution plates 2 mg – 30 µm. The retained phenolic compounds were eluted with 75 µl of acetone/ultrapure water/acetic acid solution 70/29/5.0 v/v/v and directly analyzed by UHPLC-MS/MS, using a Waters TQD mass spectrometer coupled to a Waters Acquity UPLC (Milford, MA, USA). Phenolic acids, flavonols, flavan-3-ols and resveratrol metabolites were separated at 30 °C on a Waters Acquity UPLC HSS T3 column (2.1 × 100 mm, 1.8 µm) using 0.2% acetic acid in ultrapure water and acetonitrile as A and B mobile phases respectively, with a gradient elution starting from 5% to 35% B in 10 min, raised to 80% B over 0.10 min and held for 0.90 min, and then reset to initial conditions. The mobile phase flow rate was set at 0.4 ml min⁻¹, and the injection volume was 2.5 µl. Following the separation, the flow was introduced by negative mode electrospray ionization (ESI) into the mass spectrometer with the following parameters: capillary voltage, 2.75 kV; source temperature, 140 °C; cone gas flow rate, 80 l h⁻¹ and desolvation gas flow rate, 800 l h⁻¹; desolvation temperature, 400 °C. Anthocyanins were separated at 30 °C on a Supelco Titan C18 column (2.1 × 100 mm, 1.9 µm) using mobile phases A and B (10% acetic acid in ultrapure water and acetonitrile, respectively) by gradient elution starting from 5% to 35% B in 10 min, raised to 80% B over 0.10 min and held for 0.90 min, and then reset to initial conditions. The mobile phase flow rate was set at 0.4 ml min⁻¹, and the injection volume was 2.5 µl. The MS/MS analysis was carried out in the positive ionization mode with the following parameters: capillary voltage, 2.5 kV; source temperature, 150 °C; cone gas flow rate, 50 l h⁻¹; desolvation gas flow rate, 800 l h⁻¹; desolvation temperature, 350 °C. Cone voltage and collision energy were optimized for each compound. Phenolic metabolites were identified by comparison with retention time and molecular ions of individual standards, and the quantification was conducted using their calibration curves. Remaining metabolites, for which standards were not available, were identified based on fragmentation information described in the literature and quantified using the calibration curve of their aglycone or the most similar phenolic structure. Parent/product ion pairs (MRM transitions) of identified phenolic metabolites are listed in Table 2.

2.4. Data analysis

Plasma concentrations of phenolic metabolites following acute or chronic administration of fruit extracts were compared using the Welch’s t-test (correcting for unequal variance) when data were assumed to be normally distributed, or using the Mann–Whitney test otherwise (GraphPad Prism 6.05, La Jolla, CA, USA). Similarly, the effect of treatments on phenolic metabolite concentrations in plasma and feces was analyzed for pair comparison using the Welch’s t-test or the Mann–Whitney test. Multiple comparisons were performed using one-way analysis of variance (ANOVA) or the non-parametric Kruskal–Wallis test based on whether data followed a normal distribution or not. Differences were considered to be significant at p < 0.05. Hierarchical clustering analysis (HCA) of phenolic metabolites detected in plasma and feces was carried out using MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/) on log-transformed data, with Euclidean distance measure and Ward clustering algorithm.

3. Results

3.1. Identified metabolites of blueberry and grape phenolic compounds

As presented in Table 1, treatment with a blueberry-grape extract provided flavan-3-ols (catechin, epicatechin), proanthocyanidins, gallic acid and resveratrol from the grape extract, as
well as anthocyanins, flavonols (quercetin) and chlorogenic acid from the blueberry extract.

Following supplementation of mice with either B, G or BG, a total of 32 phenolic metabolites were identified, 19 of which were detected in plasma and 27 in feces. These metabolites are listed in Table 2. Their concentrations quantified in plasma were detected in plasma and 27 in feces. These metabolites are identified in the plasma and/or feces of all supplemented animals, such as (+)-catechin, (-)-epicatechin, resveratrol and absorbable dimers/trimers of proanthocyanidins, for animals supplemented with B or BG (methylated, glucuronidated and/or sulfated metabolites). Some native phenolic compounds were also identified in plasma and/or feces, such as (+)-catechin, (-)-epicatechin, resveratrol and absorbable dimers/trimers of proanthocyanidins, for animals supplemented with G or BG, as well as chlorogenic acid and glycosides of anthocyanins for animals supplemented with B or BG. Furthermore, several phenolic acids, mostly resulting from the microbial degradation of native phenolic compounds, were identified in the plasma and/or feces of all supplemented animals, such as dihydroxycinnamic acid, ferulic acid, gallic acid, protocatechuic acid, vanillic acid and derivatives of phenylpropionic and phenylacetic acids. Likewise, microbial metabolites derived from catechins and proanthocyanidins (γ-valerolactones) and from resveratrol (dihydro-resveratrol) were mostly identified in the feces of mice supplemented with G or BG.

### Table 2 Phenolic metabolites identified in plasma and feces of mice after the administration of fruit extracts

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Standard for quantification</th>
<th>MRM</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1 (±)-Catechin</td>
<td>Epicatechin</td>
<td>289 &gt; 109</td>
<td>Plasma (G, BG), feces (G, BG)</td>
</tr>
<tr>
<td>M2 (−)-Epicatechin</td>
<td>Epicatechin</td>
<td>289 &gt; 109</td>
<td>Plasma (G, BG), feces (G, BG)</td>
</tr>
<tr>
<td>M3 Catechin glucuronide</td>
<td>Epicatechin</td>
<td>465 &gt; 289</td>
<td>Plasma (G, BG), feces (G, BG)</td>
</tr>
<tr>
<td>M4 Catechin sulfate</td>
<td>Epicatechin</td>
<td>369 &gt; 289</td>
<td>Plasma (G, BG), feces (G, BG)</td>
</tr>
<tr>
<td>M5 Catechin glucuronide sulfate</td>
<td>Epicatechin</td>
<td>545 &gt; 289</td>
<td>Plasma (G, BG), feces (G, BG)</td>
</tr>
<tr>
<td>M6 Methyl catechin glucuronide</td>
<td>Epicatechin</td>
<td>479 &gt; 303</td>
<td>Plasma (G, BG), feces (G, BG)</td>
</tr>
<tr>
<td>M7 Methyl catechin sulfate</td>
<td>Epicatechin</td>
<td>383 &gt; 303</td>
<td>Plasma (G, BG), feces (B, G, BG)</td>
</tr>
<tr>
<td>M8 Methyl catechin glucuronide sulfate</td>
<td>Epicatechin</td>
<td>559 &gt; 289</td>
<td>Feces (G, BG)</td>
</tr>
<tr>
<td>M9 B-type propanidin dimers</td>
<td>Epicatechin</td>
<td>577 &gt; 289</td>
<td>Plasma (G, BG), feces (B, G, BG)</td>
</tr>
<tr>
<td>M10 B-type propanidins trimer</td>
<td>Epicatechin</td>
<td>865 &gt; 578</td>
<td>Feces (G, BG)</td>
</tr>
<tr>
<td>M11 Hydroxyphenyl-γ-valerolactone</td>
<td>Gallic acid</td>
<td>191 &gt; 147</td>
<td>Feces (G, BG)</td>
</tr>
<tr>
<td>M12 Dihydroxyphenyl-γ-valerolactone</td>
<td>Gallic acid</td>
<td>207 &gt; 163</td>
<td>Plasma (G, BG), feces (B, G)</td>
</tr>
<tr>
<td>M13 Resveratrol</td>
<td>Resveratrol</td>
<td>403 &gt; 227</td>
<td>Plasma (G, BG)</td>
</tr>
<tr>
<td>M14 Resveratol glucuronide</td>
<td>Resveratrol</td>
<td>307 &gt; 227</td>
<td>Feces (G, BG)</td>
</tr>
<tr>
<td>M15 Resveratol sulfate</td>
<td>Resveratrol</td>
<td>229 &gt; 187</td>
<td>Feces (G, BG)</td>
</tr>
<tr>
<td>M16 Dihydro-resveratrol</td>
<td>Resveratrol</td>
<td>477 &gt; 301</td>
<td>Plasma (B, BG), feces (B, G, BG)</td>
</tr>
<tr>
<td>M17 Quercetin glucuronide</td>
<td>Quercetin</td>
<td>381 &gt; 301</td>
<td>Feces (B, BG)</td>
</tr>
<tr>
<td>M18 Quercetin sulfate</td>
<td>Quercetin</td>
<td>556 &gt; 301</td>
<td>Feces (B, G, BG)</td>
</tr>
<tr>
<td>M19 Quercetin glucuronide sulfate</td>
<td>Quercetin</td>
<td>449 &gt; 303</td>
<td>Feces (G, BG)</td>
</tr>
<tr>
<td>M20 Cyanidin 3-glucoside</td>
<td>Malvidin 3-glucoside</td>
<td>463 &gt; 331</td>
<td>Feces (B, BG)</td>
</tr>
<tr>
<td>M21 Malvidin 3-arabinoside</td>
<td>Malvidin 3-glucoside</td>
<td>465 &gt; 303</td>
<td>Feces (B, BG)</td>
</tr>
<tr>
<td>M22 Delphinidin 3-glucoside</td>
<td>Malvidin 3-glucoside</td>
<td>479 &gt; 317</td>
<td>Plasma (B, BG), feces (B, G)</td>
</tr>
<tr>
<td>M23 Petunidin 3-glucoside</td>
<td>Malvidin 3-glucoside</td>
<td>493 &gt; 331</td>
<td>Plasma (B, BG), feces (B, G)</td>
</tr>
<tr>
<td>M24 Malvidin 3-glucoside</td>
<td>Malvidin 3-glucoside</td>
<td>493 &gt; 331</td>
<td>Plasma (B, BG), feces (B, G)</td>
</tr>
<tr>
<td>M25 3,4-Dihydroxyphenyl propionic acid</td>
<td>3,4-Dihydroxyphenyl propionic acid</td>
<td>181 &gt; 59</td>
<td>Feces (B, G, BG)</td>
</tr>
<tr>
<td>M26 3,4-Dihydroxyphenyl acetic acid</td>
<td>3,4-Dihydroxyphenyl acetic acid</td>
<td>167 &gt; 123</td>
<td>Feces (B, G, BG)</td>
</tr>
<tr>
<td>M27 Chlorogenic acid</td>
<td>Chlorogenic acid</td>
<td>353 &gt; 191</td>
<td>Plasma (B, G, BG), feces (B, G)</td>
</tr>
<tr>
<td>M28 Dihydroxyxanic acid</td>
<td>Caffeic acid</td>
<td>179 &gt; 79</td>
<td>Plasma (B, G, BG)</td>
</tr>
<tr>
<td>M29 Ferulic acid</td>
<td>Ferulic acid</td>
<td>193 &gt; 134</td>
<td>Plasma (B, G, BG)</td>
</tr>
<tr>
<td>M30 Gallic acid</td>
<td>Gallic acid</td>
<td>169 &gt; 79</td>
<td>Plasma (B, G, BG)</td>
</tr>
<tr>
<td>M31 Protocatechuic acid</td>
<td>Protocatechuic acid</td>
<td>153 &gt; 53</td>
<td>Plasma (B, G, BG), feces (B, G, BG)</td>
</tr>
<tr>
<td>M32 Vanillic acid</td>
<td>Vanillic acid</td>
<td>167 &gt; 152</td>
<td>Plasma (B, G, BG)</td>
</tr>
</tbody>
</table>

B: blueberry extract, G: grape extract, BG: blueberry–grape extract.

3.2. Chronic vs. acute blueberry–grape co-supplementation: plasma concentrations of phenolic compounds

The total plasma content of phenolic compounds in mice with acute or chronic supplementation (one administration vs. 15 daily administrations, respectively) with B, G or BG is presented in Fig. 2. No difference in circulating phenolic concentration was observed between single and repeated administration of individual extracts B (5 and 6 μM, respectively) and G (157 and 198 μM, respectively). In contrast, following the chronic supplementation of mice with their combination (BG), the circulating phenolic concentration was doubled (350 vs. 166 μM for acute supplementation, p = 0.0033). Among the 19 metabolites identified in the plasma of mice supplemented with BG (Table 3), 9 were found in significantly higher concentration following chronic supplementation compared to acute supplementation (from 1.8- to 3.4-fold): catechin glucuronide, dihydroxyxanic acid, methyl catechin glucuronide, quercetin glucuronide, methyl catechin sulfate, malvidin 3-glucoside, epicatechin, ferulic acid and catechin glucuronide sulfate.
3.3. Blueberry–grape combination vs. blueberry or grape supplementation: comparison of plasma concentrations of phenolic compounds

A hierarchical clustering of plasma concentrations of the 19 phenolic metabolites analyzed following the repeated administration of B, G or BG in mice is presented in Fig. 3. Only traces of ferulic and vanillic acids were detected in plasma before supplementation (D0t0). Except for dihydroxyphenyl-γ-valerolactone, no significant concentrations of phenolic metabolites were detected in plasma prior to the last gavage (D15t0), highlighting their complete elimination from circulation in less than 24 h. As visualized on the heatmap, the clustering of phenolic metabolites reflects their origin, i.e., conjugated and microbial metabolites of catechins and resveratrol, as well as dimers of proanthocyanidins from G; conjugated quercetin, chlorogenic acid and anthocyanins from B; and phenolic acids from B and G. No difference was observed in circulating concentrations of phenolic metabolites from G, whether G was administered with B or alone. On the other hand, as shown in the inset graphs in Fig. 3, phenolic metabolites from B were found in significantly higher concentrations (from 3.0- to 5.5-fold) in the plasma of mice supplemented with BG in comparison with B alone: quercetin glucuronide (30 vs. 10 nM, *p = 0.0238), malvidin 3-glucoside (36 vs. 8 nM, *p = 0.0076), chlorogenic acid (2 vs. 0.4 µM, *p = 0.0095) and petunidin 3-glucoside (8 vs. 2 nM, *p = 0.0110). Interestingly, smaller but still significant increases (2.4- to 2.8-fold) in plasma concentrations were also observed following acute supplementation of BG in comparison with B alone for malvidin 3-glucoside (11 vs. 5 nM, *p = 0.0007), chlorogenic acid (0.9 vs. 0.3 µM, *p = 0.0219) and petunidin 3-glucoside (3 vs. 1 nM, *p = 0.0008).

3.4. Blueberry–grape combination vs. blueberry or grape supplementation: comparison of the fecal content of phenolic compounds

A hierarchical clustering of fecal concentrations of the 27 phenolic metabolites analyzed following the repeated administration of B, G or BG in mice is presented in Fig. 4. Only traces

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>BG acute administration</th>
<th>BG chronic administration</th>
<th>Fold change (chronic/acute)</th>
<th>Statistical test</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)-Catechin</td>
<td>444 ± 79</td>
<td>821 ± 227</td>
<td>3.3</td>
<td>T-W</td>
<td>ns</td>
</tr>
<tr>
<td>(−)-Epicatechin</td>
<td>1105 ± 186</td>
<td>3701 ± 1058*</td>
<td>3.4</td>
<td>T-W</td>
<td>0.0374</td>
</tr>
<tr>
<td>Catechin glucuronide</td>
<td>92 348 ± 14 664</td>
<td>169 357 ± 33 929*</td>
<td>1.8</td>
<td>T-W</td>
<td>0.0334</td>
</tr>
<tr>
<td>Catechin sulfate</td>
<td>21 159 ± 6 503</td>
<td>54 063 ± 21 491</td>
<td>ns</td>
<td>M-W</td>
<td>ns</td>
</tr>
<tr>
<td>Catechin glucuronide sulfate</td>
<td>3293 ± 1454</td>
<td>11 236 ± 3750**</td>
<td>3.4</td>
<td>T-W</td>
<td>0.060</td>
</tr>
<tr>
<td>Methyl catechin glucuronide</td>
<td>27 938 ± 2447</td>
<td>72 475 ± 9901***</td>
<td>2.6</td>
<td>T-W</td>
<td>0.0014</td>
</tr>
<tr>
<td>Methyl catechin sulfate</td>
<td>4854 ± 1101</td>
<td>13 432 ± 4035*</td>
<td>2.8</td>
<td>T-W</td>
<td>0.0259</td>
</tr>
<tr>
<td>B-type procyanidin dimers</td>
<td>1298 ± 271</td>
<td>2384 ± 797</td>
<td>ns</td>
<td>M-W</td>
<td>ns</td>
</tr>
<tr>
<td>Dihydroxymethyl-γ-valerolactone</td>
<td>174 ± 85</td>
<td>1369 ± 856</td>
<td>ns</td>
<td>T-W</td>
<td>ns</td>
</tr>
<tr>
<td>Resveratrol glucuronide</td>
<td>87 ± 18</td>
<td>120 ± 45</td>
<td>ns</td>
<td>M-W</td>
<td>ns</td>
</tr>
<tr>
<td>Quercetin glucuronide</td>
<td>12 ± 3</td>
<td>30 ± 7*</td>
<td>2.6</td>
<td>T-W</td>
<td>0.0036</td>
</tr>
<tr>
<td>Petunidin 3-glucoside</td>
<td>3 ± 0</td>
<td>8 ± 2</td>
<td>ns</td>
<td>T-W</td>
<td>ns</td>
</tr>
<tr>
<td>Malvidin 3-glucoside</td>
<td>11 ± 1</td>
<td>36 ± 11*</td>
<td>3.2</td>
<td>T-W</td>
<td>0.0463</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>874 ± 180</td>
<td>1962 ± 784</td>
<td>ns</td>
<td>T-W</td>
<td>ns</td>
</tr>
<tr>
<td>Dihydroxycinnamic acid</td>
<td>2541 ± 627</td>
<td>6035 ± 1574*</td>
<td>2.4</td>
<td>T-W</td>
<td>0.0353</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>45 ± 6</td>
<td>151 ± 31***</td>
<td>3.3</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Gallic acid</td>
<td>9324 ± 1581</td>
<td>10 635 ± 2464</td>
<td>ns</td>
<td>T-W</td>
<td>ns</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>484 ± 103</td>
<td>965 ± 300</td>
<td>ns</td>
<td>M-W</td>
<td>ns</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>256 ± 49</td>
<td>526 ± 146</td>
<td>ns</td>
<td>T-W</td>
<td>0.0033</td>
</tr>
</tbody>
</table>

| Total (µM)                          | 166 ± 25                | 350 ± 72***                | 2.1                         | T-W             | 0.0033  |

Data are displayed as mean of replicates ± SEM (control group: *n = 6*, treated groups: *n = 10*). ***p < 0.005, **p < 0.01 and *p < 0.05 vs. acute administration of the blueberry–grape extract. Arrows indicate increase fold change in plasma concentration for chronic vs. acute exposure to the blueberry–grape extract. T-W: T-test with Welch correction, M-W: Mann–Whitney test, ns: not significant.
of microbial phenolic metabolites protocatechuic acid, 3,4-dihydroxyphenyl acetic acid and 3,4-dihydroxyphenyl propionic acid were detected in feces prior to the supplementation (D0). The heatmap shows the clustering of phenolic metabolites according to their detection in fecal samples of mice treated with B, G or both extracts. No difference was found in fecal concentrations of phenolic metabolites from G, whether G was administered with B or alone. On the other hand, as shown in the inset graphs in Fig. 4, and in accordance with the previous observations in plasma, phenolic metabolites from B were found in significantly lower concentrations (from 2.9- to 6.3-fold) in the feces of mice supplemented with BG in comparison with B alone: quercetin glucuronide (147 vs. 431 pmol g⁻¹, p = 0.0499), petunidin 3-glucoside (50 vs. 157 pmol g⁻¹, p = 0.0064), protocatechuic acid (11 vs. 35 nmol g⁻¹, p < 0.0001), cyanidin 3-glucoside (14 vs. 51 pmol g⁻¹, p = 0.0005), chlorogenic acid (8 vs. 38 nmol g⁻¹, p < 0.0001) and delphinidin 3-glucoside (14 vs. 87 pmol g⁻¹, p < 0.0001). As an example, comparative MRM profiles of chlorogenic acid analyzed in mouse plasma and feces are presented in Fig. 5, showing different peak intensities between samples from animals supplemented with blueberry or blueberry–grape extracts.

4. Discussion

Numerous factors affect the bioavailability of ingested phenolic compounds, including their poor absorption, extensive metabolism and interactions with gut microbiota.20–22 Only 5–10% of the total phenolic intake is estimated to be absorbed in the small intestine, while the remaining 90–95% accumulates in the colon and is subjected to microbial degradation.23 Therefore, phenolic compounds have recently been shown to display prebiotic action by modulating the gut microbial community,23–25 thereby indirectly affecting their own biodegradation.19 The bioavailability of phenolic compounds is also strongly affected by phase I, II and III metabolism, taking place both in the intestine and the liver.5 Phase I reactions (oxidation, reduction and hydrolysis) aim to expose or add a functional group to facilitate phase II reactions, catalyzing conjugation with hydrophilic endogenous molecules, resulting in
methylated, glucuronidated and/or sulfated conjugates. Phenolic compounds and their metabolites are substrates for transmembrane transporters such as the ATP-binding cassette (ABC) transporters, and hence effluxed back into the intestinal lumen for ultimate excretion from the body. Enhancing the low bioavailability of phenolic compounds is therefore a major focus of studies, and some strategies have been recently discussed, such as designing specific synergies between phenolic compounds to favor their absorption.

In the present study, the repeated daily administration of the blueberry–grape extract to mice for 15 days was found to significantly improve the bioavailability (defined as plasma concentrations measured 30 minutes after ingestion) of key phenolic compounds by up to 3.4-fold, in comparison with a single acute administration. This increase in the plasma concentration of phenolic metabolites was not explained by accumulation over time as they were not detected in circulation prior to the last gavage, and thus were completely removed from the circulation in less than 24 h. A similar improvement in the bioavailability of phenolic compounds over time was previously reported following oral administration of a grape seed extract (GSPE) to rats over a period of 10 days with dose escalation. The authors proposed as an explanation for this observation that GSPE either induced a modulation of expression and/or activity of specific intestinal cell transporters, or an alteration of lower intestine microbial ecology (most likely by proanthocyanidins) resulting in a modified capacity for colonic fermentation of phenolic compounds. Interestingly, in the present work, enhancement in the bioavailability of phenolic compounds over time was

Fig. 4 Hierarchical clustering analysis heatmap of phenolic metabolites identified in feces of mice following their chronic supplementation with fruit extracts. Each row represents a detected metabolite and each column an animal of the study. Blue and red tiles, respectively, indicate a lower or higher intensity of metabolite concentration in feces than the mean of all samples. Metabolites assignment (M1–M32) is listed in Table 2. Inset graphs represent blueberry phenolic metabolites whose concentration in feces was significantly decreased following 15 days of blueberry–grape co-supplementation. Data are displayed as mean of replicates ± SEM (n = 10). B: blueberry extract, G: grape extract, BG: blueberry–grape extract. ***p < 0.005, **p < 0.01 and *p < 0.05 vs. blueberry extract alone.

This journal is © The Royal Society of Chemistry 2016 Food Funct.
observed with the blueberry–grape combination but not with individual extracts, indicating a positive interaction between blueberry and grape phenolic components. Indeed, blueberry phenolic compounds were more bioavailable when co-ingested with grape phenolic compounds, as indicated by the increase in the plasma concentrations of blueberry phenolic metabolites (up to 5.5-fold) matched by an equivalent decrease in their fecal concentrations (up to 6.3-fold) in mice chronically supplemented with blueberry (B), grape (G) or blueberry–grape (BG) extracts. An increase in the plasma concentrations of blueberry phenolic metabolites was also observed in animals acutely supplemented with the blueberry–grape extract, indicating that the potentiation of absorption of blueberry phenolic compounds by grape phenolic compounds occurred at the first administration. However, the bioavailability of grape phenolic compounds was not affected by the blueberry–grape co-supplementation, suggesting that such interactions may not be reciprocal.

Although the notion that interactions may occur between phytochemicals of co-ingested foods is generally well recognized and often cited, it is actually poorly documented and seldom quantified in vivo. It has been widely described in regard to food–drug interferences, but only a few studies actually reported such interactions leading to a potentiated bioavailability of phytochemicals in vivo. For example, biochanin A, an isoflavone with chemopreventive properties was found to be 3-fold more bioavailable in rats when orally administered together with quercetin and epigallocatechin gallate (EGCG). Curcumin bioavailability was reported to be increased by 154% in rats and by 2000% in humans when co-administered with piperine, an alkaloid from black pepper. Moreover, piperine has been shown to enhance the absorption of EGCG in mice, with a 1.3-fold increase in plasma concentration associated with a slower appearance of the compound in the feces, in comparison with mice treated with EGCG only. In these studies, the improved bioavailability was attributed to a combined inhibition of phase I/II enzymes and ABC efflux transporters. Indeed, phenolic compounds are not only substrates for enzymes taking part in xenobiotic metabolism but also act as their regulators, resulting in an enhancement or limitation in the bioavailability of co-ingested compounds. The mechanistic hypotheses put forward in these few studies using isolated phytochemicals may certainly be transposed to plant extracts and whole foods. Indeed, the present work reports actual interactions between phenolic compounds in complex mixtures, with the grape extract enhancing the absorption of blueberry phenolic compounds. Likewise, our previous study reported an enhanced absorption of phenolic compounds from a blend of strawberry and cranberry extracts when co-administered in rats with a quercetin-rich onion extract. These findings strongly suggest that the presence of a concentrated phenolic compound from the first extract (+)-catechin/(-)-epicatechin from the grape extract in the present study) may saturate efflux transport and thus favor the absorption of phenolic compounds of low concentration from a co-ingested extract (such as anthocyanins and chlorogenic acid from the blueberry extract). Although the mechanisms underlying this potentiation are not yet elucidated, and in accordance with the previous studies, we suggest that the improved bioavailability observed in the present work results from a probable competition between phenolic compounds as substrates/inhibitors of detoxification enzymes and/or efflux transporters. The modulatory effect of phytochemicals on detoxification processes can occur either directly or through an interaction with transcription factors involved in the regulation of an organism’s biological responses to xenobiotics such as the aryl hydrocarbon receptor (AHR).

5. Conclusion
Enhancing the low bioavailability of phenolic compounds and their metabolites is believed to be a key strategy to improve their therapeutic effects. In the present work, a grape extract

![Fig. 5](image_url) Representative MRM signals of chlorogenic acid (353 > 191) from the chemical standard, and the plasma/feces specimen from mice supplemented with blueberry (B), grape (G) or blueberry–grape (BG) extracts.
was found to potentiate the bioavailability of phenolic compounds from a blueberry extract in mice. This study provides for the first time in vivo evidence of positive interactions occurring between co-ingested phytochemicals from complex mixtures, resulting in improved bioavailability of phenolic compounds. These findings therefore highlight a new promising strategy for the development of functional foods, i.e. the opportunity to design specific combinations of phenolic compounds that could be beneficial for human health.

Acknowledgements

Financial support was provided by Nutra Canada and Atrium Innovations through the Neurophenols Consortium http://www.neurophenols.org/index.php/en/. The Neurophenols Consortium is a Europe–North America research collaboration dedicated to the research and development of natural ingredients and products to prevent age-related cognitive decline in humans and pets. The Consortium brings together scientists in the fields of phytochemistry, neuroscience, psychology and nutrition with companies specialized in the development of active ingredients and food supplements. The specific aims of the program are to characterize and formulate fruit extracts from blueberry and grape and to evaluate their safety and efficacy in pre-clinical and clinical trials. Other grants to FC from the Canada Foundation for Innovation (10307) and the Alzheimer Society Canada (ASC 0823) also supported this work. FC is a Fonds de recherche du Québec – Santé (FRQ-S) senior research scholar. ADP was supported by a scholarship from the Alzheimer Society Canada. The authors thank Véronique Richard for her technical support during the experiments.

References

23 F. Cardona, C. Andrés-Lacueva, S. Tulipani, F. J. Tinaholes and M. I. Queipo-Ortuño, Benefits of polyphenols on gut...


Dietary polyphenol supplementation prevents alterations of spatial navigation in middle-aged mice. Front Behav Neurosci. 2016.
Spatial learning and memory deficits associated with hippocampal synaptic plasticity impairments are commonly observed during aging. Besides, the beneficial role of dietary polyphenols has been suggested as potential functional food candidates to prevent this memory decline. Indeed, polyphenols could potentiate the signaling pathways of synaptic plasticity underlying learning and memory. In this study, spatial learning deficits of middle-aged mice were first highlighted and characterized according to their navigation patterns in the Morris water maze task. An eight-week polyphenol-enriched diet, containing a polyphenol-rich extract from grape and blueberry (PEGB; from the Neurophenols Consortium) with high contents of flavonoids, stilbenes and phenolic acids, was then successful in reversing these age-induced effects. The use of spatial strategies was indeed delayed with aging whereas a polyphenol supplementation could promote the occurrence of spatial strategies. These behavioral results were associated with neurobiological changes: while the expression of hippocampal calmodulin kinase II (CaMKII) mRNA levels was reduced in middle-aged animals, the polyphenol-enriched diet could rescue them. Besides, an increased expression of nerve growth neurotrophic factor (NGF) mRNA levels was also observed in supplemented adult and middle-aged mice. Thus these data suggest that supplementation with polyphenols could be an efficient nutritional way to prevent age-induced cognitive decline.

Keywords: age, hippocampus, berries, polyphenols, learning and memory, strategy, navigation

INTRODUCTION

Aging is associated with cognitive impairments and increased risks of neurodegenerative disorders such as Alzheimer’s disease which may contribute to the loss of the ability to live independently (Evans et al., 1989). In this context it seems paramount to better understand the effects of aging on learning and memory and to develop new strategies to prevent or counteract the age-associated memory decline.

The hippocampus is one of the brain structures involved in spatial learning and memory which is particularly affected during aging (Erickson and Barnes, 2003). These impairments have been observed not only in rodents (Barnes, 1979; Markowska et al., 1989; Gallagher and Rapp, 1997; Bach et al., 1999) but also in monkeys (Lai et al., 1995; Gallagher and Rapp, 1997) and in humans (Uttl and Graf, 1993; Wilkniss et al., 1997; Gazova et al., 2013). Indeed, in a
real-space adapted version of the Morris water maze for humans, it has been shown that the profile of allocentric (world-centered, hippocampus-dependent) spatial navigation learning, but not egocentric navigation, is particularly altered in adults over 70 (Gazova et al., 2013). The age-related profile of spatial navigation has also been studied in mice in the star maze and in the Morris water maze (Martel et al., 2007; Fouquet et al., 2011). Although it is accepted that aged mice (over 17 months old) abandon sequential egocentric and/or allocentric (hippocampus-dependent) strategies in favor of egocentric or cued (striatum-dependent) strategies (Kim et al., 2001; Martel et al., 2007), the training protocols (massed vs. spaced) may influence the adoption of a strategy over another. Indeed, these studies cannot fully translate the parallel and progressive acquisition of non-spatial to spatial strategies that are observed in the Morris water maze (Janus, 2004; Brody and Holtzman, 2006; Stone et al., 2011; Ruediger et al., 2012), and that depends on the interaction of multiple memory systems (Gazova et al., 2013). It is thus of interest to further analyze how aging may influence this evolution (Gil-Mohapel et al., 2013) and the differential use of non-spatial and spatial strategies by middle-aged and aged animals. The neurobiological basis for differences in navigation patterns is not fully understood but based on previous studies it is possible to hypothesize that differential activations of the hippocampus and the striatum during the learning phase could explain this evolution. Indeed, the hippocampus and the striatum must act in parallel during the acquisition of the task (Colombo et al., 2003; Martel et al., 2007).

Long-term memories are associated with molecular changes (Carew, 1996; Kandel, 2001; Sossin, 2008), such as the synthesis of new mRNAs and proteins (Martin et al., 2000; Kelleher et al., 2004; Bramham and Wells, 2007). Various signaling pathways involved in the control of de novo protein synthesis such as extracellular signal regulated kinase (ERK) (Sweatt, 2001, 2004) converge to the activation of the transcription factor cAMP-response element-binding protein (CREB) which binds the promoter regions of many genes associated with synaptic plasticity underlying spatial learning and memory (Harris and Kater, 1994; Impy et al., 1996, 1998, 2004; Pham et al., 1999; Barco et al., 2006; Alvarez and Sabatini, 2007). Hippocampal levels of phosphorylated calmodulin kinase II (CaMKII), known to regulate the phosphorylation of CREB (Dash et al., 1991), are reduced in aged animals relative to young animals. CREB also regulates the transcription of neurotrophins, such as brain derived neurotrophic factor (BDNF) or nerve growth neurotrophic factor (NGF), implicated not only in neuronal survival, outgrowth and differentiation (Finkbeiner et al., 1997; Finkbeiner, 2000; Schinder and Poo, 2000; Conkright et al., 2003; Prunusild et al., 2011) but also in the control of synaptic plasticity and long-term memory (Poo, 2001; Bramham and Messaoudi, 2005; Calabrese et al., 2009). Similarly, the use of non-spatial strategies could trigger their expression in the striatum.

There is increasing evidence of how dietary habits or nutrients may exert beneficial effects on brain aging. Among “functional foods” promoting a healthy aging, fruits and vegetables rich in polyphenols are now highly studied for their potential beneficial effects on memory (Shukitt-Hale et al., 2008; Rendeiro et al., 2009; Keen et al., 2015). Within the polyphenol family, flavonoids have been shown to ameliorate learning and memory in both animals and humans. Flavonoids, and particularly epicatechin and catechin, present in grape seeds, green tea or cocoa for example, have been shown to ameliorate learning and memory in animals (Deví et al., 2006; Haque et al., 2006; van Praag et al., 2007; Kaur et al., 2008; Asha Devi et al., 2011; Rendeiro et al., 2013b) and in humans (Dinges, 2006). Anthocyanins, present in red berries as in blueberries, have also been shown to prevent memory deficits in aged animals (Cho et al., 2003; Ramirez et al., 2005; Barros et al., 2006; Shukitt-Hale et al., 2006; Rendeiro et al., 2013b). About flavanols, quercetin has been undoubtedly the most extensively studied flavonol in their neuroprotective potential in vivo (Dajas et al., 2003, 2013; Rivera et al., 2004; Ahmad et al., 2011). Clinical studies have also observed an improvement of memory in older people with a supplementation with grape juice or with blueberry juice rich in flavonoids (Krikorian et al., 2010a,b, 2012). Moreover, flavonoid consumption has been associated with better cognitive performance in an epidemiological study over 10 years (Letenneur et al., 2007). Stilbenes and more precisely resveratrol, found mainly in grapes and wine, have displayed beneficial effects on learning and memory in animals (Abraham and Johnson, 2009; Dal-Pan et al., 2011; Harada et al., 2011; Kodali et al., 2015) and in humans (Witte et al., 2014). A naturally dimethylated analog of resveratrol, pterostilbene found in blueberries (Aiyer et al., 2012) and grapes (Adrian et al., 2000), exhibits similar biological activities (Rimando et al., 2002) and have been reported to be effective in reversing cognitive deficits in aged rats (Joseph et al., 2008). Phenolic acids are a very large polyphenols family as most of them can be the results of the microbial metabolism of other polyphenols (Margalef et al., 2014). Phenolic acids have also displayed beneficial effect to reverse memory impairments (Yan et al., 2001; Vauzour et al., 2007). Interestingly, polyphenols are not only able to impact on learning and memory during aging but they can also modulate neuronal signaling cascades altered with aging. Thereby, polyphenols may act on ERK/CREB pathway involved in synaptic plasticity and long-term potentiation (Williams and Grayar, 2004; Schroeter et al., 2007; Vauzour et al., 2007; Rendeiro et al., 2009). Indeed, flavonoid supplementations can modulate specific signaling kinases like CaMKII (Abraham and Johnson, 2009; Rendeiro et al., 2009), and ERK (Rendeiro et al., 2009, 2013a), controlling the activation of CREB and the increased expression of BDNF (Abraham and Johnson, 2009; Rendeiro et al., 2009, 2013a; De Nicóló et al., 2013) and of NGF (De Nicóló et al., 2013) in the hippocampus. Some studies have also reported that polyphenols, among them flavanols, anthocyanins or resveratrol, are found in the brain tissue after treatment. Thereby, polyphenols may act on ERK/CREB pathway involved in synaptic plasticity and long-term potentiation (Williams and Grayar, 2004; Schroeter et al., 2007; Vauzour et al., 2007; Rendeiro et al., 2009). Indeed, flavonoid supplementations can modulate specific signaling kinases like CaMKII (Abraham and Johnson, 2009; Rendeiro et al., 2009), and ERK (Rendeiro et al., 2009, 2013a), controlling the activation of CREB and the increased expression of BDNF (Abraham and Johnson, 2009; Rendeiro et al., 2009, 2013a; De Nicóló et al., 2013) and of NGF (De Nicóló et al., 2013) in the hippocampus. Some studies have also reported that polyphenols, among them flavanols, anthocyanins or resveratrol, are found in the brain tissue after oral ingestion (Abd El Mohsen et al., 2002, 2006; Asensi et al., 2002; Andres-Lacueva et al., 2005; Williams et al., 2008; Prasain et al., 2009; Juan et al., 2010; Milbury and Kalt, 2010). The possible existence of specific polyphenol binding sites at the cellular plasma membrane level in the rat brain has been suggested (Han et al., 2006).
While flavonoids and stilbenes, present in berry extracts, have potential properties against age-related cognitive decline, their specific benefits on spatial navigation patterns are unknown.

In this study, we investigated the effects of age and of a polyphenol-rich extract from grape and blueberry (PEGB; from the Neurophenols Consortium) supplementation, with high contents of flavonoids, on hippocampus-dependent learning and memory processes. First, the learning and memory deficits observed in middle-aged mice were better characterized according to their navigation patterns in the Morris water maze, then, the effectiveness of dietary polyphenols in the prevention of age-associated memory decline was assessed and the neurobiological correlates underlying these effects were investigated.

**MATERIALS AND METHODS**

**Animals and Diet**

For this study 20 adult (6-week old) and 24 middle-aged (16-month old) male C57Bl/6J mice were purchased from Janvier (France). They were singly housed in a room with a constant airflow system, controlled temperature (21–23°C), and a 12 h light/dark cycle. Mice were given *ad libitum* access to food and water. Naïve mice were randomly divided into four experimental groups (Figure 1A). One group of adult mice (*n* = 10) and one of middle-aged mice (*n* = 12) were fed with a control diet (INRA Jouy-en-Josas, France), whereas the two other groups of adult mice (*n* = 10) and middle-aged mice (*n* = 12) received a PEGB-enriched diet (INRA Jouy-en-Josas, France) containing 500 mg of PEGB/kg bw/day (provided by the Neurophenols Consortium).

The composition of the PEGB-enriched pellets was the same as the control pellets, except for the polyphenol content (Table 1). Diets started as soon as mice arrived in the laboratory (i.e., at the age of 6 weeks for adult mice and 16 months for middle-aged mice) and continued throughout the entire experiment (8 weeks). Thereby, at the end of the experiment adult mice were 3.5 months old and middle-aged mice were 18 months old. The PEGB is a powder made of grape (*Vitis vinifera* L.; Activ’Inside, France) and blueberry (*Vaccinium angustifolium*; NutraCanada, Canada) extracts containing specific polyphenols with low molecular weight (flavanols monomers and oligomers, flavonols, anthocyanins, phenolic acids and resveratrol) formulated in a unique ratio of molecules. The 500 mg of PEGB/kg bw/day dose was determined by a literature review and proportions for each polyphenol class of interest were adjusted in the mix. This specific ratio of polyphenols remains confidential. All experiments were performed in accordance with the European Communities...
TABLE 1 | Composition of the control and the PEGB-enriched diets.

<table>
<thead>
<tr>
<th>Components</th>
<th>Control diet</th>
<th>PEGB-enriched diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrochloric casein</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Corn starch</td>
<td>40</td>
<td>45</td>
</tr>
<tr>
<td>Sucrose</td>
<td>29.9</td>
<td>24.39</td>
</tr>
<tr>
<td>Cellulose</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Peanut oil</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Rapeseed oil</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Mineral compound 102</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Vitamin compound 102</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>without vitamine A</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>+ DL methionine</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>+ Vitamin A 5 UI/g</td>
<td>SU/l/g</td>
<td>SU/l/g</td>
</tr>
<tr>
<td>PEGB</td>
<td>0</td>
<td>0.51</td>
</tr>
</tbody>
</table>

PEGB extract was introduced at 0.51% in the diet pellets.

Council Directives (86/609/EEC) and the French national Committee (87/848) recommendations, and have been approved by the Animal Care and Use Committee of Bordeaux under the No. 5012085-A.

Body Weight and Food Intake
Body weight and food intake were monitored weekly during the 8-week supplementation period.

Spatial Learning and Reference Memory in the Morris Water Maze

Training Phase
Six weeks after the beginning of the diets, spatial learning and memory were assessed in a Morris water maze (150 cm in diameter, 50 cm-high) filled with white water (22°C) and surrounded with distal extramaze cues (Figure 1B). Before being trained, animals were handled 1 min a day for 2 days. Mice were then familiarized to water and swimming during two familiarization days (day-1 and day-2) where they had to find a visible platform in the center of a small pool (60 cm diameter) surrounded with curtains (three consecutive trials a day; 60 s-cut-off). On day 0, to evaluate visuomotor deficits, mice were given six trials (90 s-cut-off) to find a visible platform pointed out with a cue in the Morris water maze that was surrounded with white curtains. During the training sessions (days 1–4), animals were required to locate the submerged platform by using distal extramaze cues. They were trained for six trials a day (90 s-cut-off) with an intertrial interval of 5 min for four consecutive days. In order to facilitate spatial learning, mice were introduced from four different starting points, in a randomized daily order. The speed, the latency and the distance to reach the platform as well as the swim path of each trial were recorded by Imetronics videotracking system (France). The daily swim path efficiency was calculated as the ratio of the shortest possible length to the effective swim path length.

Probe Test
Seventy two hours after the last training session, the platform was removed from the pool and spatial memory was evaluated for 60 s. The percentage of distance traveled in the four quadrants was recorded using the SMART system (San Diego Instruments). The quadrant where the platform was located during training is referred to as target quadrant. Additionally, the number of annulus crossings and the mean proximity to the platform were assessed during this test as reliable measures of probe test performance (Maei et al., 2009).

Analysis of Navigation Strategies
For each trial of the training phase, the navigation path was analyzed from the replay of the videotracking system (Imetronics, France) and assigned to one of the eleven strategies by two experimenters, blind to the groups. The categorization scheme (see Figure 4A) was adapted from those developed previously (Brody and Holtzman, 2006; Garthe et al., 2009; Stone et al., 2011; Ruediger et al., 2012). These strategies were divided into two main categories: non-spatial vs. spatial strategies. Non-spatial strategies included first “global search” strategies: “peripheral looping” (persistent swimming around the outer 15 cm of the pool, including thigmotaxis), “random” (searching the entire tank, >75% surface coverage), “circling” (swimming in tight circles, possibly with some net directional movement), and then “local search” strategies: “scanning” (searching restricted to a limited, often central, portion of the tank, >15% and <75% of surface coverage), “chaining” (circular swimming at an approximately fixed distance greater than 15 cm from the wall), “repeated incorrect” (swimming in a precise direction that does not contain the platform and repeat the same trajectory several times), and “focal incorrect” (searching intently a small portion of the tank that does not contain the platform). Spatial strategies included “repeated correct” (swimming in direction of the platform and repeat the same trajectory several times), “focal correct” (swimming and searching intently in the zone containing the platform), “spatial indirect” (swimming indirectly to the platform with eventually 1–2 loops) and “spatial direct” (swimming directly to the platform).

Tissue Preparation
Ninety minutes after the probe test, mice were euthanized by cervical dislocation and decapitated. Brains were rapidly removed and hippocampi were dissected and frozen with liquid nitrogen before being stored at −80°C until assay.

Quantitative Real-Time PCR Analysis
Hippocampal and striatal gene expression were measured as previously described (Touyarot et al., 2013). RNA extraction was conducted using TRIzol reagent kit (Invitrogen, France) according to the manufacturer’s instructions. The integrity of the purified RNA was verified using the RNA 6000 Nano LabChip kit in combination with the 2100 Bioanalyzer (Agilent Technologies, France). The concentrations of RNA were determined by using a Nanodrop ND-1000 (Labtech, France). Using oligoDT and random primers (Promega, France), cDNA was synthesized from 1 µg of RNA with ImPromII reverse transcriptase (Promega, France) according to the manufacturer’s protocol. Briefly, 1 µg
of total RNA mixed with RNAse (Promega) and DNase (Roche) was incubated at 37°C. Then, OligoT and random primers were added for incubation at 70°C. The reverse transcriptase reaction was performed at 42°C for 60 min in a final volume of 20 µL.

The real-time PCR was performed using the LightCycler 480 system with a 96-well format (Roche Diagnostics, Germany) in a volume of 20 µL, containing 1 × LightCycler 480 SYBR Green I Master solution, 0.5 µM of each primer and 6 µL of cDNA. The forward and reverse primer sequences and the amplicon size Actin, NGF, BDNF, CaMKII, ERK1 and ERK2 are summarized in Table 2. Actin was used as the reference gene since its expression level was unaffected in our experimental conditions. The following program started with an initial denaturation step for 10 min at 95°C, then an amplification for 40 cycles (10 s denaturation at 95°C, 6 s annealing at 62°C, and 10 s extension at 72°C), finally a melting curve analysis was run. In order to verify the specificity and the identity of the amplified products: (1) the melting curve analysis showed a single melting peak after amplification; and (2) amplified products for each gene were verified by sequencing with the Big Dye Terminator v1.1. (Applied Biosystems) and analyzed on a ABI3130 sequencer (Applied Biosystems).

Quantification data were analyzed using the LightCycler 480 Relative Quantification software (version 1.5). In order to compensate for differences in target and reference gene amplification efficiency, either within or between experiments, this software provides a calibrator-normalized relative quantification including a PCR efficiency correction. Therefore, the results are expressed as the target/reference ratio divided by the target/reference ratio of the calibrator. In our case, the calibrator was chosen among the adult mice.

### Statistical Analysis

Statistical analyses were performed using StatView® (SAS Institute Inc., USA). Graphical representations were performed using GraphPad Prism®. Results were considered significantly

![FIGURE 2](image-url)

**FIGURE 2** | Effects of 8 weeks of PEGB supplementation on food intake and body weight gain. (A) Aging increases food intake but PEGB does not have any impact on it (age effect ***p < 0.0001** by 2-way ANOVA; n = 9–11 per group). (B) Adult mice gain more weight during the 8-week supplementation than middle-aged mice but PEGB does not impact on their weight gain (age effect ***p < 0.0001** by 2-way ANOVA; n = 9–11 per group).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Nucleotide sequence 5′-3′</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
</table>
| Actin     | F: AAAACGCCAGCTCAAGAAGTCGCAGTCG  
R: AGATGCCGAAAGGATATTACTGC | 220 |
| NGF       | F: ATCAAGGGGCAAGGAGGTGACAG  
R: GAGTCTGAGTGTGGAGTCAQG | 143 |
| BDNF      | F: AACCATAAGGACGCGGACTTG  
R: TTGACTGAGCTGACATCACCC | 51 |
| ERK1      | F: TCCCCATAGGCGTGATGAG  
R: CCATTCCAGAACGGTCTACCAGA | 102 |
| ERK2      | F: TTCCTAAATGCTGACTCCAAAG  
R: AAGTCTGGCAAATCTGATGCAAC | 179 |
| CaMKII    | F: AGATCTGGCAACCTGGAATGAC  
R: AGCTGATCCGAGTATAAGGGCAGTGC | 194 |

Actin; NGF, Nerve Growth Factor; BDNF, Brain-Derived Neurotrophic Factor; ERK, Extracellular signal-Regulated Kinases; CaMKII, Calmodulin-dependent protein kinase II.
different when \( p < 0.05 \). All results are expressed as mean ± SEM. Food intake, body weight gain, probe test comparisons (such as the traveled distance in target quadrant, the swim speed during the cued task and the probe test, the platform annulus crossings and the proximity to the platform) and PCR data were analyzed using a 2-way ANOVA (with two factors: age and diet) followed by a post hoc Fisher PLSD test when appropriate. Spatial learning, search strategy, swim speed during the learning phase, swim path efficiency, probe test analysis were analyzed using a 3-way ANOVA with repeated measures (age, diet and days or quadrants) followed by a post hoc Fisher PLSD test when appropriate. Probe test comparisons of each group with chance level (25%) were performed with a one-sample \( t \)-test. For the distance traveled in different quadrants during the probe test, intra-group comparisons were performed using a one-way ANOVA followed by a Dunnett’s multiple comparison test.

During the cued phase of the behavioral task, one middle-aged control mouse was excluded from the experiment because it was floating and did not look for the platform. During the training phase, five mice (one adult control mouse, two adult supplemented mice, one middle-aged control mouse and one middle-aged supplemented mouse) were excluded from the experiment because they may not have learned that there was an escape platform, therefore they were not searching for it. They were identified thanks to an intra-group outlier analysis on the distance and the latency to reach the platform (mean ± 2 SD).

RESULTS

Effects of Age and PEBG-Enriched Diet on Food Intake and Weight Gain

Food intake and weight gain were measured along the 8 weeks of diet exposure. Middle-aged mice consume more food than adult mice whatever the diet as revealed by the 2-way ANOVA on average food intake (effect of age \( [F_{1,32} = 84.451, p < 0.0001] \), no effect of diet \( [F_{1,32} = 0.724, \text{n.s.}] \) and no age \( \times \) diet interaction \( [F_{1,32} = 12.387, \text{n.s.}] \)) (Figure 2A).

Adult mice that are completing their full growth gain more weight over the 8 weeks than middle-aged mice regardless of the diet. Indeed, a 2-way ANOVA on average body weight gain revealed an age effect \( (F_{1,32} = 20.958, p < 0.0001) \), no diet effect \( (F_{1,32} = 1.587, \text{n.s.}] \), and no age \( \times \) diet interaction \( (F_{1,32} = 0.007, \text{n.s.}] \) (Figure 2B).

Effects of Age and PEBG-Enriched Diet on Spatial Learning and Memory

To acquire the procedural aspects of the task such as swimming and climbing onto the platform and to evaluate visuo-motor capabilities, mice were first trained to find a visible platform in the Morris water maze without distal cues. Middle-aged mice swam slower than adult mice, under a control or a PEBG-enriched diet as revealed by an ANOVA performed on the swim speed over the trials in the cued task (age effect \( [F_{1,32} = 4.325, p < 0.05] \), with no effect of diet \( [F_{1,32} = 0.354, \text{n.s.}] \) and no interaction age \( \times \) diet \( [F_{1,32} = 0.959, \text{n.s.}] \)) (data not shown). The same observation has been made during the learning phase (age effect \( [F_{1,32} = 17.758, p < 0.001] \), with no effect of diet \( [F_{1,32} = 0.089, \text{n.s.}] \), no interaction age \( \times \) diet \( [F_{1,32} = 1.642, \text{n.s.}] \) and no day effect \( [F_{1,96} = 2.648, \text{n.s.}] \)) (Figure 3A). As the latency to reach the platform is dependent on the swimming speed, the distance covered to reach the platform was chosen as a more appropriate measure to show the acquisition rate for this cued task and for spatial learning.

In this way, all groups had similar visual capabilities and did not show any impairment in the cued version of the Morris water maze task. Indeed, all groups traveled similar distances to reach the visible platform because the 2-way ANOVA on the distance to reach the visible platform indicated no effect of age \( (F_{1,32} = 2.935, \text{n.s.}] \), no effect of diet \( (F_{1,32} = 0.012, \text{n.s.}] \), and no age \( \times \) diet interaction \( (F_{1,32} = 0.905, \text{n.s.}] \) (data not shown).

From day 1, mice were trained in the spatial version of the Morris water maze to test the effects of age and PEBG on spatial learning and memory. The four groups of mice traveled significantly less and less distance over the 4 days (Figure 3B), indicating that all groups learned the platform location (day effect \( [F_{3,96} = 39.862, p < 0.0001] \)). However, middle-aged mice traveled significantly longer distance than adult mice to find the platform, revealing spatial learning deficits (ANOVA: age effect \( [F_{1,32} = 5.256, p < 0.05] \)). Moreover, mice that are under a PEBG-enriched diet showed better performance than mice under a control diet (ANOVA: diet effect \( [F_{1,32} = 4.182, p < 0.05] \)). Interestingly, there was no interaction age \( \times \) diet, suggesting that this diet effect was observed not only in middle-aged supplemented mice which performed as well as adult control mice \( [F_{1,32} = 2.137, \text{n.s.}] \), but also in adult mice that are under the PEBG-enriched diet and seem to perform better especially on the first day of training. On the last training day, all groups traveled similar distance to reach the platform (Figure 3B). These results were confirmed by the analyses on the swim path efficiency. Indeed, the ANOVA similarly revealed a day effect \( (F_{1,96} = 44.837, p < 0.0001) \) an age effect \( (F_{1,96} = 8.551, p < 0.01) \) and a trend for an interaction age \( \times \) diet \( (F_{1,32} = 3.976, p = 0.0547) \) and for a diet effect \( (F_{1,32} = 3.206, p = 0.0828) \) (Supplementary Figure 1). Indeed, on the first learning trials, all mice may complete the task by chance (as they have not constructed any cognitive map yet): this could hide the effects of polyphenols in the path efficiency.

Spatial memory was assessed during the probe test. As the swim speed is still affected by age on this trial (Figure 3C), the traveled distance has been chosen as the most appropriate parameter. One sample test comparing to the chance level (25%) revealed that all groups swam significantly more distance in the target quadrant (adult control: \( p < 0.05 \); adult supplemented: \( p < 0.05 \); middle-aged control: \( p < 0.05 \); middle-aged supplemented: \( p < 0.01 \) ) (Figure 3D), revealing that 72 h after the last training session, the four groups remembered the location of the platform. Besides, a 2-way ANOVA performed on the percentage of distance traveled in the different quadrants revealed no quadrant \( \times \) age interaction \( (F_{1,96} = 0.395, \text{n.s.}] \) but a quadrant \( \times \) diet interaction \( (F_{4,96} = 2.935, p < 0.05) \).
FIGURE 3 | Effects of 8 weeks of PEGB supplementation on spatial learning and memory. (A) Swim speed during learning is reduced with aging but not affected by the supplementation with PEGB (age effect: ***p < 0.001 by 3-way ANOVA; n = 9–11 per group). (B) Distance covered to reach the hidden platform over the four consecutive days of spatial learning (trials are averaged for each training day). Middle-aged mice exhibit longer distance compared to adult mice to reach the platform during the training sessions while mice fed with the PEGB-enriched diet travel less distance to reach the platform than mice under a control diet (day effect p < 0.0001; age effect *p < 0.05; diet effect #p < 0.05 by 3-way ANOVA with repeated measures). (C) Swim speed during the probe test is reduced in middle-aged mice (age effect: ***p < 0.0001). (D) Percentage of distance traveled in quadrants during the probe test. The dotted line corresponds to chance level (25%). All groups can remember the platform location traveling preferentially more distance in the target quadrant and the PEGB-enriched diet slightly ameliorates memory performance ($p < 0.05, $$p < 0.01 vs. chance level by one sample t-test. *p < 0.05, **p < 0.01, ***p < 0.001 compared to QW-Target by One-way ANOVA and Dunnett’s multiple comparison test; n = 9–11 per group). (E) The number of crossings of the platform annulus is not impacted by age nor by PEGB. (F) The mean proximity to the platform is similar for all groups.
FIGURE 4 | Effects of 8 weeks of PEGB supplementation on search strategies during spatial learning in the Morris water maze. (A) Representative path patterns that reflect "non-spatial" (blue) or "spatial" (red) strategies used to reach the hidden platform (filled gray circle). Operational definitions of individual strategies are described in the method section. (B) Search strategies used during learning. Non-spatial strategies are represented in blue colors and spatial strategies in red colors. (C) All groups show an evolution of the use of non-spatial to spatial strategies (day effect: \(p < 0.0001\)). An age effect and a diet effect are also observed (age effect: \(\ast\ast\ast p < 0.0001\); diet effect: \(\ast\ast p < 0.01\)). (D) Adult mice exhibit more use of spatial strategies than middle-aged mice. Moreover mice fed with the PEGB-enriched diet performed more spatial strategies than mice under the control diet (age effect: \(\ast\ast\ast p < 0.0001\); diet effect: \(\ast\ast\ast p < 0.01\) by 3-way ANOVA; \(n = 9–11\) per group).
interaction \((F_{1,96} = 3.513, p < 0.05)\), suggesting that PEGB may improve spatial memory. A one-way ANOVA realized for each group revealed a quadrant effect for the supplemented adult mice \((F_{1,32} = 9.465, p < 0.001)\), the control middle-aged mice \((F_{1,32} = 5, 051, p < 0.01)\) and the supplemented middle-aged mice \((F_{1,32} = 9.532, p < 0.001)\). Surprisingly, no quadrant effect was revealed for the adult mice under a control diet \((F_{1,32} = 2.244, n.s.)\) as they preferentially traveled within two adjacent quadrants (the target west and the south quadrants). However, as previously described, adult control mice traveled more than 30% of the total distance in the target quadrant. However, according to additional analyses no differences were revealed by the between-group ANOVA for the number of annulus crossings and the mean proximity to the platform (Figures 3E,F). Thus, the PEGB-enriched diet only slightly improves memory performance in the spatial version of the Morris water maze.

**Effects of Age and PEGB-Enriched Diet on Strategies During Spatial Learning**

In order to better characterize the age-induced spatial learning deficits, revealed with the conventional parameters, and whether dietary polyphenols could be beneficial, the navigation path of each trial was qualitatively analyzed (Figures 4A,B). The detailed analysis of navigation path revealed that control mice progressively applied different search strategies along the learning phase. Usually, consistent with previous reports (Janus, 2004; Ruediger et al., 2012), mice first adopt non-spatial strategies, beginning with global search (random swim) and then local search strategies (scanning and chaining). The strategies “focal incorrect” and “repeated incorrect” categorized as non-spatial strategies are in fact intermediate, suggesting that mice may use distal cues in an erroneous manner and that their cognitive map is not fully acquired. Then, spatial strategies (repetition of a direct path, indirect search, focal search, direct swim) appear. However, it is of note that control mice do not always go through all strategies. The increase of the use of spatial strategies evolves continuously but not linearly over the trials and correlates with a decreased average distance to reach the platform.

An evolution from non-spatial to spatial strategies over the learning days of the four groups is observed. Indeed, more and more trials are solved using one of the four spatial strategies (3-way ANOVA on navigation strategies: day effect \([F_{3,96} = 51.242, p < 0.0001]\)) (Figure 4C). Moreover, this analysis revealed an age effect \([F_{1,32} = 23.116, p < 0.0001]\) and a diet effect \([F_{1,32} = 10.345, p < 0.01]\). These data suggest that middle-aged mice take more time to adopt spatial strategies than adult mice (only 50% of middle-aged control mice and 60% of middle-aged supplemented used a spatial strategy on the first day) and that mice under the PEGB-enriched diet are more prone to use spatial strategies than mice under the control diet. This diet effect is principally observed in the first learning day for adult mice where an ANOVA revealed a significant diet effect \([F_{1,32} = 8.114, p < 0.001]\) (Figure 4C): indeed, 100% of the adult mice consuming PEGB were able to use one of the spatial strategies within the six first trials whereas only 62.5% of adult mice under the control diet did so (data not shown).

This is confirmed when all trials over the four learning days are considered (Figure 4D): indeed, compared to adult mice, middle-aged mice used less spatial strategies to complete the task (age effect \([F_{1,32} = 23.116, p < 0.0001]\)). Besides, when mice are under a PEGB-enriched diet, they used more spatial strategies than mice under a control diet (diet effect \([F_{1,32} = 10.345, p < 0.01]\)).

**Effects of Age and PEGB-Enriched Diet on Hippocampal and Striatal Gene Expression**

To study the neurobiological correlates associated with the age-induced spatial deficits and the polyphenol-induced behavioral improvements, the mRNA levels of proteins involved in synaptic plasticity were measured (Figure 5). In the hippocampus a significant increase in NGF mRNA levels was observed both in adult and middle-aged mice fed with PEGB-enriched diet \((F_{1,24} = 4.645, p < 0.05)\) without being affected by aging (age effect \([F_{1,24} = 0.604, n.s.]\)) (age × diet effect \([F_{1,24} = 0.239, n.s.]\)) (Figure 5A). However, no effect of age or diet was found on the hippocampal BDNF mRNA levels (Figure 5B). The ANOVA on ERK1 mRNA levels revealed an age effect \((F_{1,25} = 7.806, p < 0.01)\) with an increased expression in middle-aged animals (Figure 5C) whereas no effect of age or diet was observed on the ERK2 mRNA expression (Figure 5D). Moreover, the hippocampal CaMKII mRNA expression is decreased in middle-aged mice and could be offset by a supplementation with polyphenols. The ANOVA on hippocampal CaMKII expression revealed an age × diet interaction \((F_{1,28} = 4.877, p < 0.05)\) and the post hoc Fisher PLSD test showed a significant difference between the mRNA levels observed in middle-aged control mice and those of the adult control mice \((p < 0.05)\) and of the middle-aged supplemented mice \((p < 0.05)\) (Figure 5E).

In order to verify the non-involvement of the striatum in the use of spatial strategies, the expression profile of the same mRNAs was assessed. However, in our conditions, striatal NGF and BDNF mRNA levels were too low to be quantified (data not shown) and no difference in the levels of striatal CaMKII mRNA expression was observed showing that the changes observed in the hippocampus were specific to this structure (Figure 5F).

**DISCUSSION**

In the present study, we have shown for the first time that a supplementation with a PEGB ameliorates the profile of spatial navigation learning in the Morris Water maze and by this way slightly improves memory of middle-aged mice. Indeed, we evidenced with the categorization of navigation strategies that middle-aged mice take more time to adopt spatial strategies to solve the Morris water maze task. Besides, a supplementation in polyphenols can facilitate the use of spatial strategies by adult and
FIGURE 5 | Effects of PEGB supplementation on hippocampal (Hpc) and striatal (St) plasticity-related gene expression. (A,B) NGF mRNA expression increases significantly in the hippocampus of supplemented animals (diet effect \( \# p < 0.05 \)) but no differences induced by age or diet are found in hippocampal brain derived neurotrophic factor (BDNF) mRNA levels. (C,D) Hippocampal ERK1 mRNA levels are increased with age but not with polyphenols (age effect \( ** p < 0.01 \)). No changes in hippocampal ERK2 levels are induced by age nor diet. (E,F) Age decreases CaMKII mRNA levels in the hippocampus which are restored by the PEGB-enriched diet \( (*) p < 0.05 \). However, no differences are observed in the striatum \( (n = 6–10 \text{ per group}) \).
middle-aged mice. Moreover, these learning impairments are associated with decreased hippocampal CaMKII mRNA levels whereas performance improvements can be linked to the restoration of hippocampal CaMKII mRNA levels and to an increased hippocampal NGF expression with the PEGB supplementation.

The Morris water maze is considered as a test of spatial learning and memory (Morris et al., 1982; Morris, 1984) but this task can also be solved by non-spatial strategies. Consistent with previous reports (Morris, 1984; Wolder and Lipp, 2000; Janus, 2004; Ruediger et al., 2012) adult control mice completed the task using different search strategies along the learning phase, starting from non-spatial strategies, then intermediate strategies like “focal incorrect” and “repeated incorrect” to switch progressively to spatial strategies (notably focal search around the platform and direct swim to the platform). The increase of the use of spatial strategies evolves continuously but not linearly over the trials as mice may alternate non-spatial and spatial strategies over consecutive trials before using almost exclusively spatial strategies. Moreover, we found that this navigation pattern evolution is influenced by age and diet. It is known that the Morris water maze task is particularly sensitive to cognitive deficits related to aging (Frick et al., 1995; Lindner, 1997; Wolff et al., 2002; de Fiebre et al., 2006). Indeed, age would specifically affect spatial allocentric and sequential egocentric strategies in mice (Fouquet et al., 2011). In this study, we have demonstrated for the first time that the evolution from non-spatial to spatial strategies during learning that is particularly delayed with age is promoted with a supplementation in polyphenols.

The analysis of the navigation strategies allows a more thorough knowledge on the acquisition of spatial memory and permits to put on the fore subtle learning differences. Here, longer escape distance to reach the platform traveled by middle-aged mice does not reflect a constant random search of the entire surface area of the pool, which would have indicated a complete lack of spatial learning abilities. It rather shows that some middle-aged mice rely on persistent performance of an alternative strategy that is successful to reach the escape platform but that could appear less efficient if the conditions change. Thus middle-aged mice took more trials to switch to spatial strategies; indeed, their first use of spatial strategies would appear later and most of them would not exclusively use spatial strategies even on the last training day. Indeed, the adoption of spatial strategies is already compromised in 12-month old mice (Gil-Mohapel et al., 2013). In the current study, we used 16–18 month old mice as middle-aged mice as they represent a suitable model with mild cognitive deficits for a 2-month nutritional approach (Pepeu, 2004; Bonhomme et al., 2014).

Here, we show that the PEGB can improve spatial learning in both adult and middle-aged mice. Unlike the middle-aged control mice, middle-aged mice that were fed the PEGB-enriched diet learned as quickly as adult control mice. The extract supplementation could thus prevent from the occurrence of age-related learning deficits. The different families of polyphenols present in this extract are known to be effective on cognitive functions in particular flavanols (van Praag et al., 2007; Rendeiro et al., 2013a), anthocyanins (Rendeiro et al., 2009, 2013a; Shan et al., 2009; Gazova et al., 2013) and resveratrol (Abraham and Johnson, 2009; Dal-Pan et al., 2011; Harada et al., 2011; Kodali et al., 2015). However, this is the first nutritional intervention with a mix of different polyphenols at low doses that shows a rescue effect on those specific memory deficits.

In this study, it could be hypothesized that hippocampal impairments in middle-aged mice impede the use of spatial strategies: middle-aged mice would thus solve the task using preferentially striatum-dependent strategies (Packard and McGaugh, 1996; Devan et al., 1999; Colombo et al., 2003; Kelleher et al., 2004; Teather et al., 2005). Indeed, when the hippocampus is altered, the spatial navigational strategies may not be possible or too costly. On the contrary, polyphenols could preserve the involvement of the hippocampus so that hippocampus-dependent strategies are promoted and age-related deficits reduced.

The neurobiological basis for differences in navigation patterns is not fully understood but it could be hypothesized that differential activations of the hippocampus and the striatum during the learning phase could explain this evolution. Indeed, evidence supports the view that the hippocampus and the striatum act in parallel during the acquisition of various tasks (Colombo et al., 2003; Gazova et al., 2013). Indeed, sustained hippocampal expression of Fos is found in mice and rats that predominantly use allocentric strategies (Passino et al., 2002; Colombo et al., 2003) and c-Jun-immunoreactive cells were observed in the striatum of rats that acquired a cued task (Teather et al., 2005). Phosphorylated CREB immunoreactivity (pCREB-IR) is also increased in the hippocampus of rats that use an allocentric spatial strategy to solve a radial arm maze task, whereas pCREB-IR is increased in the striatum of rats that use an egocentric response strategy (Colombo et al., 2003).

The hippocampus dysfunction during aging could particularly be due to reduced persistence and magnitude of long-term potentiation in hippocampal neurons (Barnes, 1979; Barnes and McNaughton, 1985; Deupree et al., 1991; Barnes et al., 1992; Moore et al., 1993; Geinisman et al., 1995; Rosenzweig et al., 1997; Calhoun et al., 1998; Smith et al., 2000). Polyphenols have been shown to impact on different neuronal signaling pathways involved in synaptic plasticity and long term potentiation (Williams and Grayar, 2004; Schroeter et al., 2007; Vauzour et al., 2007; Rendeiro et al., 2009) and particularly on the modulation of specific signaling pathways involved in learning and memory: our results are partially consistent with these studies. Indeed, CaMKII expression that decreases during aging can be recovered by an 8-week polyphenol supplementation. This effect could be attributed to the flavanols, provided mainly from grapes, because a catechin supplementation in aged mice has been shown to restore hippocampal expression level of CaMKII (Abraham and Johnson, 2009) whereas a blueberry supplementation, richer in anthocyanins, failed to
restore CaMKII activation in aged rats (Rendeiro et al., 2009). Contrary to what might have been expected, we did not observe modifications in ERK2 mRNA expression, whereas there was an increase in ERK1 mRNA expression in middle-aged mice whatever the diet. No modification in mRNA or total protein levels have been described up to now. However, the activation of ERK1/2 has been shown to decrease with aging and to be normalized with polyphenols from blueberry (Rendeiro et al., 2009, 2013a). Moreover, despite a nutritional approach dealing with low doses of polyphenols, the PEGB used in the present study also permitted to slightly increase hippocampal NGF mRNA expression in both adult and middle-aged mice. Such an increase in NGF expression has previously been associated with an improvement of memory performance particularly in aged rodents (Deupree et al., 1991; Woolf et al., 2001). It could thus be hypothesized that the effects of the polyphenols are supported by this NGF mRNA level increase (De Nicoló et al., 2013) but it may not be the unique factor underlying these effects. However, no modification in BDNF mRNA expression has been observed after an 8-week supplementation in polyphenols although a decrease in BDNF and pro-BDNF mRNA and proteins levels have been reported during normal aging (Phillips et al., 1991; Michalski and Fahnestock, 2003; Peng et al., 2005; Calabrese et al., 2013) and an increase in BDNF levels could have been observed as previously (Rendeiro et al., 2013a).

In order to evidence the differential activation of the hippocampus and the striatum during learning and to determine how polyphenols could impact on the choice of the used strategy, we have also evaluated gene expression in the striatum, focusing on BDNF, NGF and CaMKII expressions. As NGF and BDNF mRNA expressions were too low to be quantifiable in the striatum, their modulation may not be responsible for the observed behavioral effects. However, unlike in the hippocampus, CaMKII expression in the striatum was not modulated by age nor by polyphenols. These results suggest that the modulation of CaMKII expression is specific to the hippocampus and could be linked to the effect of age and polyphenols on the use of “spatial” strategies.

It would be interesting to know how polyphenols precisely act to modify gene expression. It has been established through the use of autoradiography, that specific phenol binding sites for resveratrol exist throughout the rat brain (Han et al., 2006). The action of various polyphenols and resveratrol analogs could be mediated by the activation of common “receptor” binding sites particularly enriched at the level of the cellular plasma membrane in the rat brain. It is though unclear what the molecular nature of those receptors might be (Barco et al., 2006). It has also been reported that the effects of resveratrol could also be linked to its interaction with sirtuins (Borra et al., 2005; Donnez et al., 2010; Sadowska-Bartosz and Bartosz, 2014). Besides, van Praag (2009) has proposed the existence of specific membrane receptors for flavonoids.

The present results show that learning alterations in the early stage of aging can be overcome with a nutritional intervention with polyphenols. Our study focused on spatial learning in mice but other potential effects of dietary polyphenols on other kinds of memory must be studied more thoroughly. Thus, polyphenols are potential food nutrients that can help in the prevention of the age-related cognitive decline (Impy et al., 2004; Andres-Lacueva et al., 2005; Schroeter et al., 2007; Shukitt-Hale et al., 2008; Abraham and Johnson, 2009; Rendeiro et al., 2009; Dal-Pan et al., 2011; Harada et al., 2011; Kean et al., 2015) and our results suggest that they can play an important role for memory early in life. Furthermore, future investigations are still needed to better determine how polyphenols act at the molecular level to modulate gene expression, which potentially leads to improved cognitive performance. As preventive strategies for healthy aging are being developed, their definition must take into account environmental factors such as nutrition to promote the maintenance of a satisfactory cognitive state in elderly subjects and to avoid -or at least delay—any evolution towards memory loss and dementia (Gómez-Pinilla, 2008; Joseph et al., 2009; van Praag, 2009; Murphy et al., 2014).

AUTHOR CONTRIBUTIONS

JB, DG, SL, VP and PL conceived and designed the experiments; JB, LS, SA and PL performed the experiments; JB, PL and SA analyzed the data; JB and PL wrote the manuscript.

ACKNOWLEDGMENTS

This work is part of the Neurophenols project selected in the 12th call for FUI (Fonds Unique Interministériel) projects. The Neurophenols Consortium is a Europe-North America research collaboration dedicated to the research and development of natural ingredients and products to prevent age-related cognitive decline in humans and pets. The Consortium brings together scientists in the fields of phytochemistry, neuroscience, psychology and nutrition with companies specialized in the development of active ingredients and food supplements. The specific aims of the program are to characterize and formulate fruit extracts from blueberry and grape, to evaluate their safety and efficacy in pre-clinical and clinical trials. The project has been supported by Bpifrance, the Aquitaine Regional Council (Conseil Régional d’Aquitaine), and by the European Regional Development Fund (ERDF). The authors would like to thank Mathieu Cadet and Philippe Birac for animal care. We are also grateful to the Neurophenols consortium and to Damien Bonhomme, Maël Guillard and Katia Touyard for their technical contribution and their comments on the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnbeh.2016.00009/abstract

Supplementary Figure 1 | Swim path efficiency during spatial learning. Swim path efficiency is only affected by age but PEGB-enriched diet tends to improve learning (age effect: **p < 0.01; diet effect: p = 0.0828; age × diet effect: p = 0.0547 by 3-way ANOVA, n = 9–11 per group).
Bensalem et al.

Polyphenols and Aging: Spatial Learning

REFERENCES

doi: 10.1016/j.pneurobio.2005.06.003
analysis in PDAPP mice before and after experimental traumatic brain injury.
Calabrese, F., Guidotti, G., Racagni, G., and Riva, M. A. (2013). Reduced
neuroplasticity in aged rats: a role for the neurotrophin brain-derived
neurobiolaging.2013.06.014
a link between stress and mood disorders. Psychoneuroendocrinology 34,
S208–S216. doi: 10.1016/j.psyneuen.2009.05.014
Mouton, P. R., et al. (1998). Hippocampal neuron and synaptophysin-positive
1016/s0197-4580(98)00098-0
5–8. doi: 10.1016/s0896-6273(00)80016-1
Antioxidant and memory enhancing effects of purple sweet potato anthocyanin
1007/bf02980027
Conkright, M. D., Guzmán, E., Flechner, L., Su, A. I., Hogenesch, J. B., and
doi: 10.1016/S1097-2765(03)00134-5
Dajas, F., Andrés, A. C., Florencia, A., Carolina, E., and Felicia, R. M.
(2013). Neuroprotective actions of flavones and flavonols: mechanisms and
Chem. 13, 30–35. doi: 10.2174/1871524911313010005
Dajas, F., Rivera, F., Blasina, F., Arredondo, F., Echeverry, C., Lafon, L.,
et al. (2003). Cell culture protection and in vivo neuroprotective capacity of
performances are selectively enhanced during chronic caloric restriction
1371/journal.pone.0016581
response element-binding protein is activated by Ca2+/calmodulin- as well as
doi: 10.1073/pnas.88.11.5061
learning and psychomotor performance of C57BL/6 mice: age sensitivity
1007/s11357-006-9027-3
(2013). Effects of olive polyphenols administration on nerve growth factor and
doi: 10.1016/j.nut.2012.11.007
correlates with in vitro potentiation in young and aged Fischer 344 rats. Brain
Res. 554, 1–9. doi: 10.1016/0006-8993(91)90164-q
Devan, B. D., McDonald, R. J., and White, N. M. (1999). Effects of medial and
lateral caudate-putamen lesions on place- and cue-guided behaviors in the
1016/s0166-4328(98)00107-7
(GSPE) and antioxidant defense in the brain of adult rats. Med. Sci. Monit. 12,
BR124–BR129.
1097/00005344-200606001-00019

Abd El Mohsen, M., Bayele, H., Kuhnle, G., Gibson, G., Debnam, E., Kaila Srai, S.,
et al. (2006). Distribution of [3H]trans-resveratrol in rat tissues following oral
Abd El Mohsen, M. M., Kuhnle, G., Rechner, A. R., Schroeter, H., Rose, S.,
1016/s0891-5849(02)01137-1
with resveratrol reduced infection-related neuroinflammation and deficits in
working memory in aged mice. Rejuvenation Res. 12, 445–453. doi: 10.1089/rej.
2009.0888
Stilbene content of mature Vitis vinifera berries in response to UV-C elicitation.
J. Agric. Food Chem. 48, 6103–6105. doi: 10.1021/jf0009910
(2011). Quercetin protects against oxidative stress associated damages in a rat
model of transient focal cerebral ischemia and reperfusion. Neurochem. Res. 36,
1360–1371. doi: 10.1007/s11064-011-0458-6
Influence of berry polyphenols on receptor signaling and cell-death pathways:
doi: 10.1021/jf204084f
neuro.30.051606.094222
rats are found centrally and may enhance memory. Nutr. Neurosci. 8, 111–120.
doi: 10.1080/10284150500078117
Asensi, M., Medina, I., Ortega, A., Carretero, J., Baño, M. C., Obrador, E.,
et al. (2002). Inhibition of cancer growth by resveratrol is related to its
Bach, M. E., Barad, M., Son, H., Zhuo, M., Lu, Y. F., Shih, R., et al. (1999). Agerelated defects in spatial memory are correlated with defects in the late phase
of hippocampal long-term potentiation in vitro and are attenuated by drugs
that enhance the cAMP signaling pathway. Proc. Natl. Acad. Sci. U S A 96,
5280–5285. doi: 10.1073/pnas.96.9.5280
1471-4159.2006.03870.x
neurophysiological and behavioral study in the rat. J. Comp. Physiol. Psychol.
93, 74–104. doi: 10.1037/h0077579
doi: 10.1037/0735-7044.99.6.1040
loss of synaptic contacts in hippocampal field CA1. Hippocampus 2, 457–468.
doi: 10.1002/hipo.450020413
Barros, D., Amaral, O. B., Izquierdo, I., Geracitano, L., do Carmo Bassols
effects of Vaccinium berries intake in mice. Pharmacol. Biochem. Behav. 84,
229–234. doi: 10.1016/j.pbb.2006.05.001
Bonhomme, D., Pallet, V., Dominguez, G., Servant, L., Henkous, N., Lafenêtre, P.,
et al. (2014). Retinoic acid modulates intrahippocampal levels of corticosterone
in middle-aged mice: consequences on hippocampal plasticity and contextual
Borra, M. T., Smith, B. C., and Denu, J. M. (2005). Mechanism of human SIRT1
m501250200

Frontiers in Behavioral Neuroscience | www.frontiersin.org

13

February 2016 | Volume 10 | Article 9




Bensalem et al.

Polyphenols and Aging: Spatial Learning


Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2016 Bensalem, Servant, Alfos, Gaudout, Layé, Lafenetre and Pallet. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution and reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.
Short-term supplementation with a specific combination of dietary polyphenols increases energy expenditure and alters substrate metabolism in overweight subjects.
Int J Obes. 2014.
BACKGROUND AND OBJECTIVES: Impaired regulation of lipid oxidation (metabolic inflexibility) is associated with obesity and type 2 diabetes mellitus. Recent evidence has indicated that dietary polyphenols may modulate mitochondrial function, substrate metabolism and energy expenditure in humans. The present study investigated the effects of short-term supplementation of two combinations of polyphenols on energy expenditure (EE) and substrate metabolism in overweight subjects.

SUBJECTS AND METHODS: Eighteen healthy overweight volunteers (9 women, 9 men; age 35 ± 2.5 years; body mass index 28.9 ± 0.4 kg m⁻²) participated in a randomized, double-blind cross-over trial. Combinations of epigallocatechin-gallate (E, 282 mg day⁻¹) + resveratrol (R, 200 mg day⁻¹) and E + R + 80 mg day⁻¹ soyo isoflavones (S) or placebo capsules (PLA) were supplemented twice daily for a period of 3 days. On day 3, circulating metabolite concentrations, EE and substrate oxidation (using indirect calorimetry) were measured during fasting and postprandial conditions for 6 h (high-fat-mixed meal (2.6 MJ, 61.2 E% fat)).

RESULTS: Short-term supplementation of E + R increased resting EE (E + R vs PLA: 5.45 ± 0.24 vs 5.23 ± 0.25 kJ min⁻¹, P = 0.039), whereas both E + R (699 ± 18 kJ 120 min⁻¹ vs 676 ± 20 kJ 120 min⁻¹, P = 0.028) and E + R + S (704 ± 18 kJ 120 min⁻¹ vs 676 ± 20 kJ 120 min⁻¹, P = 0.014) increased 2-4 h-postprandial EE compared with PLA. Metabolic flexibility, calculated as the postprandial increase to the highest respiratory quotient achieved, tended to be improved by E + R compared with PLA and E + R + S only in men (E + R vs PLA: 0.11 ± 0.02 vs 0.06 ± 0.02, P = 0.059; E + R + S: 0.03 ± 0.02, P = 0.009). E + R + S significantly increased fasting plasma free fatty acid (P = 0.064) and glycerol (P = 0.021) concentrations compared with PLA.

CONCLUSIONS: We demonstrated for the first time that combined E + R supplementation for 3 days significantly increased fasting and postprandial EE, which was accompanied by improved metabolic flexibility in men but not in women. Addition of soy isoflavones partially reversed these effects possibly due to their higher lipolytic potential. The present findings may imply that long-term supplementation of these dosages of epigallocatechin-gallate combined with resveratrol may improve metabolic health and body weight regulation.

INTRODUCTION
Disturbances in lipid metabolism have a key role in the development of obesity, type 2 diabetes mellitus and cardiovascular disease. A mismatch between energy supply and expenditure as well as intrinsic disturbances in the capacity to adapt fuel oxidation to fuel availability (defined as metabolic inflexibility) in adipose tissue and skeletal muscle are the major causes of obesity-related complications.¹

Impairments in the lipid-buffering capacity of adipose tissue may lead to lipid accumulation in non-adipose tissues (ectopic fat deposition) in conditions where oxidative capacity is insufficient.² It is well-established that lipid accumulation in the liver and skeletal muscle is strongly associated with insulin resistance.³ Indeed, decreased fasting lipid oxidation and impaired switching between lipid and carbohydrate fuels in response to insulin, dietary stimuli or exercise has been observed in conditions of insulin resistance.¹,⁴,⁵ Underlying mechanisms for metabolic inflexibility may be a reduced mitochondrial function or capacity,⁶ although recent studies indicate that glucose disposal rather than mitochondrial dysfunction is a determinant of substrate utilization during insulin stimulation.⁷

Lifestyle interventions, aiming at reducing (saturated) fat intake and increasing physical activity, have been demonstrated to efficiently counteract disturbances in lipid metabolism and seem to improve metabolic flexibility.⁸,⁹ However, lifestyle interventions have been shown to be ineffective in about 30% of the subjects, indicating the need for additional preventive strategies.

Reversal of metabolic impairments by means of dietary supplementation may be a good strategy to increase the success of lifestyle interventions. Dietary polyphenols are natural components of fruits and vegetables that have recently been shown to alter substrate and energy metabolism. Resveratrol (R), an activator of silent mating type information regulation 2 homolog 1 (SIRT1, a member of the NAD⁺ dependent deacetylases family of sirtuins) and peroxisome proliferator-activated receptor gamma co-activator 1 alpha...
Dietary polyphenols and substrate metabolism

J Most et al

SUBJECTS AND METHODS

Subjects

Eighteen healthy, weight-stable overweight (body mass index, 25–30 kg m⁻²) Caucasian subjects (age, 20–50 years) with a normal fasting glucose (<6.1 mmol l⁻¹) and normal blood pressure (systolic blood pressure, 100–140 mm Hg; diastolic blood pressure, 60–90 mm Hg) participated in this study. Subjects were assigned to order of treatments according to a computer-generated, randomization plan (block size, n = 6). An independent researcher generated the randomization and provided the blinded supplements. Exclusion criteria were (a history of) diabetes, coagulation disorders, pulmonary, cardiovascular, hepatic, renal or gastrointestinal diseases, liver or thyroid disorders. Furthermore, subjects were excluded when using dietary supplements, among others vitamins, electrolytes or antioxidants or having a high habitual intake of caffeine (>300 mg day⁻¹), green tea (>1 cup day⁻¹) or alcohol (>20 g day⁻¹). Finally, subjects were excluded from participation if they were on a special diet, donated blood, took antibiotics, followed intense fitness training, were smokers, pregnant or lactating or were using drugs or medication interfering with the outcomes of the present study over the 3 months before the start of the study. All procedures were according to the Declaration of Helsinki, all subjects gave written informed consent for the study, which was reviewed and approved by the local Medical Ethical Committee of the Maastricht University Medical Center.

Study design

In this double-blind randomized placebo-controlled cross-over trial, subjects received two combinations of polyphenol supplements and placebo in randomized order: (1) epigallocatechin-gallate (E: 282 mg day⁻¹) + resveratrol (R: 30 mg day⁻¹); (2) E + R + soy isoflavones (S: 80 mg day⁻¹); and (3) placebo (PLA), containing partly hydrolyzed microcrystalline cellulose. Subjects consumed supplements orally for 2 days (twice daily at breakfast and dinner), and during the clinical investigation day (day 3) supplements were taken at arrival and simultaneously with the high-fat mixed meal. There was a wash-out period of at least 7 days between supplementation periods.

Clinical investigation day

Subjects were asked to maintain their habitual eating and physical activity pattern throughout the study. To standardize their eating pattern throughout the supplementation days, subjects were asked to keep a 3-day dietary record during the first supplementation period. Based on these first period records, food intake was standardized during the second and third supplementation period. Furthermore, subjects were asked to refrain from drinking alcohol and intensive exercise 48 h before the study day and to consume a low-fat, carbohydrate-rich meal at the evening before the study day. After an overnight fast of at least 12 h, subjects arrived at the laboratory (Maastricht University Medical Center) by car or bus. After ingestion with half of the daily amount of supplements, a cannula was inserted into an antecubital vein for blood sampling. Blood was sampled under fasting conditions (t = 0) and for 6 h after the intake of a liquid high-fat mixed meal (consumed within 5 min at t = 0 min) at t = 30, 60, 90, 120, 180, 240, 300 and 360 min. The liquid high-fat mixed meal had an energy content of 2.6 MJ (61% fat, 33% carbohydrate, 6% protein). Samples were immediately snap-frozen in liquid nitrogen before storage at −80 °C until further analysis. Fasting and postprandial EE and substrate oxidation were measured by indirect calorimetry, using an open-circuit ventilated hood system (Omnical, Maastricht University, Maastricht, The Netherlands).

Supplements

The test products were commercial available via Pure Encapsulations Inc. (Sudbury, MA, USA) and were encapsulated for blinding by Wellness Clinical Services (Placebo Lot 15897, Teavigo Lot 15898, Resveratrol Extra Lot: 15899, Soy Isoflavone 40 Lot: 15900). All capsules were manufactured, tested and checked in accordance to standards of EU GMP requirements. Teavigo capsules contained 94% E (141 mg per capsule), Resveratrol extra (100 mg trans-Resveratrol per capsule, combined with 46 mg grape seed polyphenols and 12.5 mg red wine polyphenols) and Soy Isoflavone 40 (40 mg soy isoflavones per capsule (100 mg)). Placebo capsules were filled with microcrystalline cellulose and encapsulated equally as the active supplements. Daily supplemented polyphenol amounts were (E, 300 mg day⁻¹; R, 150 mg day⁻¹; S, 150 mg day⁻¹) have been shown to be safe and well-tolerated in humans.

Blood analyses

Blood was sampled into pre-chilled EDTA tubes (0.2× EDTA (Sigma, Dorset, UK)). Plasma free fatty acid (FFA), triglycerides and glucose were measured with enzymatic assays on an automated spectrophotometer (ABX Pentra 400 autoanalyzer, Horiba ABX, Montpellier, France). Plasma free glyceral was measured with an enzymatic assay (Enzyte Glyceral, Roche Biopharm, Basel, Switzerland) automated on a Cobas Fara spectrophotometric autoanalyzer (Roche Diagnostics, Basel, Switzerland). Circulating plasma insulin, adiponectin and leptin concentrations were determined using commercially available radioimmunoassay (RIA) kits (Human Insulin specific RIA, Human Adiponectin RIA, Human Leptin RIA, Millipore Corporation, Billerica, MA, USA). Catecholamine analysis was performed using high-performance liquid chromatography with electrochemical detection (RECIPE Chemicals and Instruments GmbH, Munich, Germany). Plasma concentrations of inflammatory markers (interleukin-6 (IL-6), IL-8 and tumor necrosis factor-α (TNF-α)) were determined using a multiplex enzyme-linked immuno-sorbent assay (Human Proinflammatory II 4-Plex Ultra-Sensitive Kit, Meso Scale Diagnostics, Rockville, MD, USA).

Calculations

The equations of Weir30 and Frayn31 were used to calculate EE and the total rate of fat and carbohydrate oxidation, assuming that protein...
oxidation accounts for 15% of total EE. Calculations are based on measurements of VO\(_2\) consumption (l min\(^{-1}\)) and VCO\(_2\) production (l min\(^{-1}\)), averaged over 20 min for each time point.

EE (kmin\(^{-1}\)) = (3.9 × VO\(_2\)) + (1.1 × VCO\(_2\))

Carbohydrate oxidation (CHO) (gmin\(^{-1}\))

= (4.55 × VCO\(_2\)) – (3.21 × VO\(_2\)) – (2.87 × N)

Fat oxidation (FAT) (gmin\(^{-1}\)) = (1.67 × VO\(_2\)) – (1.67 × VCO\(_2\)) – (1.92 × N)

N(gmin\(^{-1}\)) = ((0.15 × EE)/17)/6.25

Macronutrient oxidation as percentages of EE: CHO/EE (%) = (CHO/(17kgJ))/EE

FAT/EE (%) = (FAT/(39kgJ))/EE

Metabolic flexibility = postprandial/RQ\(_{\text{Max}}\) – RQ\(_{\text{Fasting}}\).

Statistics

All data are given as means ± s.e.m. The postprandial response is expressed as AUC and incremental AUC (iAUC), which is calculated by the trapezoid method. IAUC values are used, when differences in resting values are different. AUC and iAUC values are given as total (0-6 h) or divided in periods of 2 h (0-2 h, 2-4 h, 4-6 h). Differences in fasting and postprandial AUC values between treatments were analyzed using analysis of variance, with gender as covariate. Only in case of a trend or a significance of a treatment (treat) effect or treatment × gender (treat × gender) interaction, post-hoc analyses with least significant difference correction were performed. Trends and significant outcomes of analysis of variance and post-hoc tests are summarized in Table 1. Statistics was done using the SPSS 19.0 (IBM Corporation, Armonk, NY, USA) for Macintosh. P < 0.05 was considered statistically significant.

RESULTS

Subjects’ characteristics

Eighteen healthy, overweight men and women volunteered to participate in this study. Subjects’ characteristics are presented in Table 2. No major differences in macronutrient composition of the diet and energy intake could be identified between subjects (3500 kcal 2 day\(^{-1}\) (14,771 kJ 2 day\(^{-1}\), 47.9 E% CHO, 36.5 E% FAT, 14.7 E% PRO).

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Total (n = 18)</th>
<th>Men (n = 9)</th>
<th>Women (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>34 ± 6.2</td>
<td>34 ± 2.3</td>
<td>34 ± 2.9</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>86.6 ± 2.9</td>
<td>91.5 ± 3.3</td>
<td>81.6 ± 1.9</td>
</tr>
<tr>
<td>Length (m)*</td>
<td>1.73 ± 0.02</td>
<td>1.76 ± 0.02</td>
<td>1.7 ± 0.02</td>
</tr>
<tr>
<td>BMI (kg m(^{-2}))</td>
<td>28.9 ± 0.4</td>
<td>29.5 ± 0.4</td>
<td>28.4 ± 0.4</td>
</tr>
<tr>
<td>Fat mass (% of total body weight)*</td>
<td>27.3 ± 1.9</td>
<td>21.6 ± 1.1</td>
<td>33 ± 1.5</td>
</tr>
<tr>
<td>Fat mass (kg)*</td>
<td>23.25 ± 1.46</td>
<td>19.7 ± 1.2</td>
<td>26.81 ± 1.23</td>
</tr>
<tr>
<td>WHR*</td>
<td>0.83 ± 0.03</td>
<td>0.92 ± 0.01</td>
<td>0.76 ± 0.02</td>
</tr>
<tr>
<td>SBP (mm Hg)*</td>
<td>121 ± 2.2</td>
<td>126 ± 1</td>
<td>116 ± 2.5</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>81 ± 1.7</td>
<td>84 ± 1.8</td>
<td>78 ± 1.4</td>
</tr>
<tr>
<td>Fgul (mmol l(^{-1}))</td>
<td>5 ± 0.09</td>
<td>5.07 ± 0.09</td>
<td>4.93 ± 0.09</td>
</tr>
<tr>
<td>Fins (mU l(^{-1})*)</td>
<td>13.58 ± 1.41</td>
<td>18.63 ± 1.32</td>
<td>12.13 ± 1.07</td>
</tr>
<tr>
<td>HOMA-IR*</td>
<td>3.45 ± 0.33</td>
<td>4.21 ± 0.31</td>
<td>2.69 ± 0.25</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; Fgul, fasted plasma glucose; Fins, fasted plasma insulin; HOMA-IR, homeostatic model assessment of insulin resistance; SBP, systolic blood pressure; WHR, waist-to-hip ratio. Values are given as means ± s.e.m. (n = 18). Statistical significant difference between gender indicated as asterisk (*) when P < 0.05.

EE and substrate oxidation

Resting EE after an overnight fast was significantly higher during E + R compared with PLA supplementation (5.45 ± 0.24 vs 5.23 ± 0.25 kcal min\(^{-1}\), P = 0.039; Figure 1a). Postprandial EE remained elevated during E + R as well as E + R + S during the mid-postprandial period compared with PLA (AUC\(_{2-4 h}\): PLA, 675 ± 20 kcal 2 h\(^{-1}\) (161 ± 5 kcal 2 h\(^{-1}\) vs E + R, 699 ± 18 (167 ± 4 kcal 2 h\(^{-1}\), P = 0.03; PLA vs E + R + S, 703 ± 18 kcal 2 h\(^{-1}\) vs 168 ± 4 kcal 2 h\(^{-1}\), P = 0.02; Figure 1a). No significant effects were observed for respiratory quotient (RQ) (Figure 1b), carbohydrate (Figure 1c) and fat oxidation (Figure 1d) in the fasting and postprandial period. In men, metabolic flexibility (calculated as the difference between fasting and highest postprandial RQ) was increased during E + R treatment as compared with PLA (E + R vs PLA, P = 0.009) and as compared with E + R + S supplementation (E + R vs E + R + S, P = 0.009) for men (Figure 1e) but not for women (Figure 1f). No differences were observed for the time point, at which the higher RQ was reached (PLA: t = 81; E + R: t = 85.0; E + R + S: t = 81.7).

Circulating metabolite concentrations

Fasting and postprandial plasma glucose (Figure 2a) and insulin concentrations (Figure 2b) were not significantly affected by supplementation of combinations of polyphenols. Fasting lactate concentrations were not different between treatments, but overall lactate response was lower during E + R + S compared with PLA (AUC\(_{0-6 h}\): 346.0 ± 17.3 vs 374.7 ± 18.8 mmol l\(^{-1}\) per 6 h, P = 0.024) (Figure 2c). No significant treatment effects were observed for plasma triglycerides concentrations (Figure 2d), whereas fasting FFA concentrations tended to be increased by E + R + S compared with PLA (508 ± 51 vs 401 ± 28 mmol l\(^{-1}\), P = 0.06). In line, FFA concentrations were higher in the postprandial phase during E + R + S compared with PLA as well as with E + R (AUC\(_{1-6 h}\): E + R, 148 ± 18; E + R + S: 163 ± 10 mmol l\(^{-1}\) 6 h\(^{-1}\), P = 0.06; E + R + S vs E + R, P = 0.02). E + R had no significant effects on plasma FFA concentrations compared with PLA (Figure 2e). Finally, fasting glycerol concentrations were elevated by E + R + S compared with PLA (E + R + S vs PLA, P = 0.02), with no differences in postprandial conditions (Figure 2f).

Circulating adipokine and norepinephrine concentrations

Fasting leptin concentrations were elevated after E + R compared with PLA and E + R + S supplementation in women but not in men, (women, PLA: 13.6 ± 1.9 vs E + R, P < 0.01), E + R: 15.6 ± 1.4, E + R + S: 13.5 ± 1.7 μg l\(^{-1}\) (vs E + R, P < 0.01) (Figure 3a). There were no significant differences in adiponectin (Figure 3b) and norepinephrine (Figure 3c) concentrations between treatments. Plasma concentrations of IL-6, IL-8 and TNF-α were not changed after polyphenol supplementation compared with PLA (Figures 3d-f).

DISCUSSION

We hypothesized that a combination of polyphenols may act additively or synergistically to enhance EE and fat oxidation. Indeed, the present study demonstrated for the first time that short-term supplementation (3 days) of E + R increased fasting and postprandial EE in humans. This was accompanied by a more pronounced increase in postprandial RQ, defined as metabolic flexibility, in men but not in women. Addition of soy isoflavones partially reversed the beneficial effects of E + R, except for the increase in postprandial EE.

No effects on resting EE have been previously found during human supplementation studies with either E or R alone in the short term\(^{12,15,18}\) or long term\(^{12,25,32-34}\). Potent EE-stimulating
effects of E in combination with caffeine have been reported
during 12 and 24 h measurements, but this effect could not be
confirmed in all studies. Nevertheless, in the former two studies,
E was only effective in combination with caffeine and not
specified to either fasting or postprandial conditions. Indeed,
3-day supplementation with E alone did not increase resting EE
in overweight volunteers, indicating that R or a synergistic effect
of both components may be responsible for the effect on resting
EE, which was found in the present study.

The potential of R to increase EE has been reported in non-
human primates after supplementation for 15 weeks. Also,
 studies in obese rodents observed that 6 and 10 weeks of R
supplementation prevented the development of obesity upon an
obesogenic diet. However, in the latter studies EE or other

Table 2. ANOVA outcome of selected variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>Period</th>
<th>ANOVA</th>
<th>Post hoc</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P-value, treatment</td>
<td>P-value, treatment × sex</td>
<td>Group</td>
</tr>
<tr>
<td>EE</td>
<td>t = 0 kJ min⁻¹</td>
<td>0.115</td>
<td>0.587</td>
<td>Total</td>
</tr>
<tr>
<td>AUC₀⁻⁶,hkJ per 6 h</td>
<td>0.122</td>
<td>0.518</td>
<td>Total</td>
<td>2046</td>
</tr>
<tr>
<td>AUC₀⁻²,hkJ per 2 h</td>
<td>0.512</td>
<td>0.193</td>
<td>Total</td>
<td>705</td>
</tr>
<tr>
<td>AUC₂⁻⁴,hkJ per 2 h</td>
<td>0.01</td>
<td>0.675</td>
<td>Total</td>
<td>676</td>
</tr>
<tr>
<td>AUC₆⁻⁴,hkJ per 6 h</td>
<td>0.331</td>
<td>0.68</td>
<td>Total</td>
<td>664</td>
</tr>
<tr>
<td>iaUC₀⁻⁴,hkJ per 6 h</td>
<td>0.133</td>
<td>0.457</td>
<td>Total</td>
<td>164</td>
</tr>
<tr>
<td>iaUC₀⁻⁴,hkJ per 2 h</td>
<td>0.035</td>
<td>0.287</td>
<td>Total</td>
<td>78</td>
</tr>
<tr>
<td>iaUC₂⁻⁴,hkJ per 2 h</td>
<td>0.226</td>
<td>0.614</td>
<td>Total</td>
<td>49</td>
</tr>
<tr>
<td>iaUC₆⁻⁴,hkJ per 2 h</td>
<td>0.226</td>
<td>0.379</td>
<td>Total</td>
<td>37</td>
</tr>
<tr>
<td>ARQ</td>
<td>0.308</td>
<td>0.026</td>
<td>Total</td>
<td>0.08</td>
</tr>
<tr>
<td>Plasma lactate</td>
<td>t = 0 mmol l⁻¹</td>
<td>0.95</td>
<td>0.414</td>
<td>Total</td>
</tr>
<tr>
<td>AUC₀⁻⁶,mmol l⁻¹ per 6 h</td>
<td>0.038</td>
<td>0.308</td>
<td>Total</td>
<td>169</td>
</tr>
<tr>
<td>AUC₀⁻²,mmol l⁻¹ per 2 h</td>
<td>0.176</td>
<td>0.313</td>
<td>Total</td>
<td>122</td>
</tr>
<tr>
<td>AUC₂⁻⁴,mmol l⁻¹ per 2 h</td>
<td>0.165</td>
<td>0.502</td>
<td>Total</td>
<td>91</td>
</tr>
<tr>
<td>AUC₆⁻⁴,mmol l⁻¹</td>
<td>0.066</td>
<td>0.163</td>
<td>Total</td>
<td>58</td>
</tr>
<tr>
<td>iaUC₀⁻⁴,mmol l⁻¹ per 6 h</td>
<td>0.114</td>
<td>0.939</td>
<td>Total</td>
<td>61</td>
</tr>
<tr>
<td>iaUC₀⁻²,mmol l⁻¹ per 2 h</td>
<td>0.159</td>
<td>0.466</td>
<td>Total</td>
<td>14</td>
</tr>
<tr>
<td>Plasm FA</td>
<td>t = 0 μmol l⁻¹</td>
<td>0.042</td>
<td>0.979</td>
<td>Total</td>
</tr>
<tr>
<td>AUC₀⁻⁶,μmol l⁻¹ per 6 h</td>
<td>0.079</td>
<td>0.564</td>
<td>Total</td>
<td>146</td>
</tr>
<tr>
<td>AUC₀⁻²,μmol l⁻¹ per 2 h</td>
<td>0.012</td>
<td>0.35</td>
<td>Total</td>
<td>35</td>
</tr>
<tr>
<td>AUC₂⁻⁴,μmol l⁻¹ per 2 h</td>
<td>0.401</td>
<td>0.535</td>
<td>Total</td>
<td>41</td>
</tr>
<tr>
<td>AUC₆⁻⁴,μmol l⁻¹</td>
<td>0.378</td>
<td>0.905</td>
<td>Total</td>
<td>70</td>
</tr>
<tr>
<td>iaUC₀⁻⁴,μmol l⁻¹ per 6 h</td>
<td>0.323</td>
<td>0.953</td>
<td>Total</td>
<td>0.9</td>
</tr>
<tr>
<td>iaUC₀⁻²,μmol l⁻¹ per 2 h</td>
<td>0.45</td>
<td>0.814</td>
<td>Total</td>
<td>12.8</td>
</tr>
<tr>
<td>Plasma glycerol</td>
<td>t = 0 mmol l⁻¹</td>
<td>0.257</td>
<td>0.945</td>
<td>Total</td>
</tr>
<tr>
<td>AUC₀⁻⁶,mmol l⁻¹ per 6 h</td>
<td>0.709</td>
<td>0.475</td>
<td>Total</td>
<td>85.7</td>
</tr>
<tr>
<td>AUC₀⁻²,mmol l⁻¹ per 2 h</td>
<td>0.316</td>
<td>0.145</td>
<td>Total</td>
<td>9.2</td>
</tr>
<tr>
<td>AUC₂⁻⁴,mmol l⁻¹ per 2 h</td>
<td>0.957</td>
<td>0.392</td>
<td>Total</td>
<td>10.1</td>
</tr>
<tr>
<td>AUC₆⁻⁴,mmol l⁻¹</td>
<td>0.815</td>
<td>0.772</td>
<td>Total</td>
<td>12.4</td>
</tr>
<tr>
<td>iaUC₀⁻⁴,mmol l⁻¹ per 6 h</td>
<td>0.445</td>
<td>0.6</td>
<td>Total</td>
<td>0.045</td>
</tr>
<tr>
<td>Plasma TAG</td>
<td>t = 0 mmol l⁻¹</td>
<td>0.403</td>
<td>0.107</td>
<td>Total</td>
</tr>
<tr>
<td>AUC₀⁻⁶,mmol l⁻¹ per 6 h</td>
<td>0.193</td>
<td>0.485</td>
<td>Total</td>
<td>714</td>
</tr>
<tr>
<td>AUC₀⁻²,mmol l⁻¹ per 2 h</td>
<td>0.343</td>
<td>0.433</td>
<td>Total</td>
<td>193</td>
</tr>
<tr>
<td>AUC₂⁻⁴,mmol l⁻¹ per 2 h</td>
<td>0.246</td>
<td>0.84</td>
<td>Total</td>
<td>271</td>
</tr>
<tr>
<td>AUC₆⁻⁴,mmol l⁻¹</td>
<td>0.274</td>
<td>0.347</td>
<td>Total</td>
<td>251</td>
</tr>
<tr>
<td>iaUC₀⁻⁴,mmol l⁻¹ per 6 h</td>
<td>0.11</td>
<td>0.13</td>
<td>Total</td>
<td>206</td>
</tr>
<tr>
<td>iaUC₀⁻²,mmol l⁻¹ per 2 h</td>
<td>0.236</td>
<td>0.453</td>
<td>Total</td>
<td>23</td>
</tr>
<tr>
<td>Plasma leptin</td>
<td>t = 0 ng l⁻¹</td>
<td>0.029</td>
<td>0.041</td>
<td>Total</td>
</tr>
<tr>
<td>AUC₀⁻⁶,ng l⁻¹ per 6 h</td>
<td>0.099</td>
<td>0.126</td>
<td>Total</td>
<td>14.4</td>
</tr>
<tr>
<td>AUC₀⁻²,ng l⁻¹ per 2 h</td>
<td>0.386</td>
<td>0.294</td>
<td>Total</td>
<td>14.9</td>
</tr>
</tbody>
</table>

Abbreviations: ANOVA, analysis of variance; AUC, area under the curve; EE, energy expenditure; E + R: epigallocatechin-gallate + resveratrol; E + R + S: epigallocatechin-gallate + resveratrol + soy isoflavones; FFA, free fatty acids; iaUC, incremental AUC; PLA, placebo; TAG, triglycerides; ARQ, metabolic flexibility. Statistical analysis of selected variables by ANOVA and post-hoc testing. *Statistically significant different from PLA. †0.028. ‡0.014. §statistically significant difference between polyphenol treatments.
measures of energy balance such as food intake and energy excretion were not measured. In contrast, we have recently demonstrated that 30-day supplementation with R alone decreased EE in healthy overweight men, mimicking the effects of caloric restriction.12

There are several possible mechanisms that may explain the increased EE as a result of short-term E+R supplementation. Rodent studies indicate that E may exert its metabolic effects through an inhibition of the noradrenaline-degrading enzyme Catechol-O-Methyl-Transferase, leading to an increased norepinephrine concentration.37,38 In the present study, norepinephrine concentrations were not affected by E+R supplementation, indicating that this cannot explain the observed increase in EE.

We cannot exclude the possibility that R has counteracted E-induced catecholamine secretion, as R may inhibit catecholamine secretion and signaling in rat cardiomyocytes and adrenal medullary cells.39,40

The present results indicate that E+R supplementation increased fasting leptin concentrations. This might have contributed to the increase in EE, as leptin infusion has been shown to stimulate EE in humans.41 In line with our findings, it has been shown that 3 days of E supplementation increased leptin expression in adipose tissue in overweight men.20 As for E supplementation alone no increase in circulating leptin concentrations have been reported in previous short-term studies, it is tempting to postulate that E and R act synergistically to enhance leptin concentration. Interestingly, leptin concentrations remained unchanged after addition of soy isoflavone to E+R supplementation, suggesting that S may impair leptin secretion. Indeed, Szkudelska et al.14 found that genistein attenuated leptin secretion by rodent adipocytes.

Finally, mitochondrial uncoupling in skeletal muscle, liver and (brown) adipose tissue might contribute to the observed increase in EE following E+R.43 Indeed, studies in rodents reported that E

---

**Figure 1.** Substrate oxidation and energy expenditure before and after a high-fat mixed meal after a 3-day polyphenol supplementation (t = 0). Values are given as means ± s.e.m. (n = 18). (a-d) Dashed line, circles: PLA; solid line, triangles: E + R; solid line, square: E + R + S; (e-f) white: PLA; grey: E + R; black: E + R + S. Statistical significance indicated as asterisk (*) when post-hoc testing P < 0.05, and as hashtag (#) when post-hoc testing P < 0.1.
or R alone stimulate expression of uncoupling proteins in tissues relevant to EE.\textsuperscript{11,44} In rodent skeletal muscle, R stimulates PGC-1\textsubscript{α}, an upstream target of uncoupling genes.\textsuperscript{11} Thus, it would be highly interesting to examine the effects of combined polyphenol supplementation on mitochondrial function in future studies. In summary, it remains to be determined whether R or the synergistic effect of R + E is responsible for the observed increase in resting EE in humans.

Metabolic flexibility, reflected by the postprandial increase in RQ, was improved by the combination of E + R compared with PLA in men but not women. A recent study performed in our laboratory provided evidence that E supplementation induced a slightly more oxidative phenotype in the skeletal muscle of obese men,\textsuperscript{20} which may improve metabolic flexibility. Additionally, we recently demonstrated that 30 days of R supplementation improved muscle mitochondrial respiration and induced a more

---

**Figure 2.** Plasma metabolite concentrations after a 3-day supplementation before and after a high-fat mixed meal (t = 0). Values are given as means ± s.e.m. (n = 18). (a–f): Dashed line, circles: PLA; solid line, triangles: E + R; solid line, square: E + R + S. Statistical significance indicated as asterisk (*) when P < 0.05; trends indicated as #, when P < 0.1.
Figure 3. Plasma adipokine, norepinephrine and cytokine concentrations after a 3-day supplementation before and after a high-fat mixed meal (t = 0). Values are given as means ± s.e.m. (n = 18). (a–h) Dashed line, circles: PLA; solid line, triangles: E + R; solid line, square: E + R + S. Statistical significance indicated as asterisk (*) when P < 0.05.

oxidative phenotype of skeletal muscle in obese men, which was accompanied by improved metabolic flexibility.\(^\text{12}\) In line, improved mitochondrial function after R or E treatment was found in rodents.\(^\text{10,11,38}\) Thus, further research has to indicate whether R, E and/or additive or synergistic effects between both components explain the short-term improvement in metabolic flexibility in men.

Interestingly, long-term supplementation of high doses of R (500 mg day\(^{-1}\), threefold higher than in the study of Timmers et al.\(^\text{12}\)) had no significant metabolic effects in young, mildly
insulin-resistant obese subjects and that R (75 mg day$^{-1}$) has no metabolic effects in non-obese women, suggesting that R is mainly beneficial in metabolically compromised states and/or at lower dosages. Therefore, we cannot exclude the possibility that the fact that we observed only an improved metabolic flexibility in men may be due to the more pronounced insulin resistance in men compared with women at baseline (HOMA, men: 4.21 ± 0.44, women: 2.69 ± 0.35). Secondly, we cannot exclude that the unaffected metabolic flexibility in women compared with men might be biased by the use of oral contraceptives and/or the phase of the menstrual cycle, which were not taken into account in the present study.

Because metabolic flexibility and mitochondrial function are closely associated with insulin sensitivity, it is tempting to postulate that prolonged combined E + R supplementation may improve insulin sensitivity. This is strengthened by long-term studies in rodents, demonstrating insulin-sensitizing properties of both E and R, which may reverse these beneficial effects more specifically, synergistic amplifications of lipolysis after combination of polyphenols (Resveratrol + Genistein) have previously been reported. Increased postprandial FFA concentrations may in turn reduce metabolic flexibility in healthy humans.

In conclusion, the present study demonstrated for the first time that short-term (3 days) supplementation of low-to-moderate dosages of E + R increased fasting and postprandial EE, which was accompanied by improved metabolic flexibility in men but not in women. Soy isoflavones partially reversed the beneficial effects of E + R, except for increased postprandial EE, possibly due to their higher lipolytic potential.

Our data indicate that the impact of polyphenol supplementation is highly dependent on the type or combination of polyphenols and on gender. Furthermore, for resveratrol supplementation it is postulated that supplementation rather prevents and reverses metabolic abnormalities in metabolically high risk subjects than affecting pathways and phenotypes of originally healthy subjects, which we suggest to account for in future studies.

Independent of gender and metabolic profile appears to be the increased EE. Assuming the increase achieved in this study by supplementing E + R maintains during long-term supplementation without counter-regulatory effects, this may result in a 3.4 kg weight loss over a period of 6 months. According to findings in the US Diabetes Prevention Program, every kilogram of weight loss reduces the risk for developing diabetes by 16%. Importantly, long-term follow-up studies should further investigate whether these short-term metabolic effects of combined polyphenol supplementation in fact translate into long-term metabolic benefits. This would yield highly important information to further optimize polyphenol supplementation, thereby improving metabolic health and preventing metabolic disease in humans.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**ACKNOWLEDGEMENTS**

We thank the study participants, as well as Jos Stegen and Wendy Sluijpers for their excellent technical support. Furthermore, we would like to thank the ALPRO foundation for funding this study. The authors’ contributions are as follows: JM executed the study; all the authors critically read the manuscript, provided suggestions for improvement and approved the final version. Supplements were kindly provided by Pure Encapsulations Inc. Trial registration: ClinicalTrials.gov, NCT01302639

**REFERENCES**

Dietary polyphenols and substrate metabolism

J Most et al
Modulation of strawberry/cranberry phenolic compounds glucuronidation by co-supplementation with onion: characterization of phenolic metabolites in rat plasma using an optimized μSPE-UHPLC-MS/MS method.

J Ag Food Chem. 2014.
Modulation of Strawberry/Cranberry Phenolic Compounds Glucuronidation by Co-Supplementation with Onion: Characterization of Phenolic Metabolites in Rat Plasma Using an Optimized μSPE–UHPLC-MS/MS Method

Stéphanie Dudonne,†,‡ Pascal Dubé,†,‡ Geneviève Pilon,†,§ André Marette,†,§ Hélène Jacques,†,|| John Weisnagel,‡ and Yves Desjardins*,†,‡

†Institute of Nutrition and Functional Foods (INAF), Laval University, 2440 Boulevard Hochegega, Québec (QC) G1V 0A6, Canada
‡Research Center of Horticulture (CRH), Laval University, 2480 Boulevard Hochegega, Québec (QC) G1V 0A6, Canada
§Department of Medicine, Quebec Heart and Lung Institute (CRIUCPQ), Laval University, 2725 Chemin Ste-Foy, Québec (QC) G1V 4G5, Canada
||Department of Food and Nutrition Sciences, Laval University, 2425 Rue de l’Agriculture, Québec (QC) G1V 0A6, Canada
¶Laval University Hospital Center (CHUL), Department of medicine, 2705 Boulevard Laurier, Québec (QC) G1V 4G2, Canada

Supporting Information

ABSTRACT: Plant phenolic compounds are suggested to exert pharmacological activities in regards to obesity and type-2 diabetes, but their mode of action is poorly understood due to a lack of information about their bioavailability. This work aimed to study the bioavailability of GlucoPhenol phenolic compounds, a strawberry–cranberry extracts blend, by characterizing plasma phenolic metabolites. A comparison was performed by co-supplementation with an onion extract. Using an optimized μSPE–UHPLC-MS/MS method, 21 phenolic metabolites were characterized, mostly conjugated metabolites and microbial degradation products of the native phenolic compounds. Their kinetic profiles revealed either an intestinal or hepatic formation. Among identified metabolites, isorhamnetin glucuronidation sulfate was found in greater amount in plasma. Three glucuronidated conjugates of strawberry–cranberry phenolic compounds, p-hydroxybenzoic acid glucuronide, catechins glucuronide, and methyl catechins glucuronide were found in higher quantities when GlucoPhenol was ingested together with onion extract (+252%, +279%, and +118% respectively), suggesting a possible induction of glucuronidation processes by quercetin. This work allowed the characterization of actual phenolic metabolites generated in vivo following a phenolic intake, the analysis of their kinetics and suggested a possible synergistic activity of phenolic compounds for improving bioavailability.

KEYWORDS: bioavailability, GlucoPhenol, μSPE, phenolic metabolites, UHPLC-MS/MS

INTRODUCTION

In recent years, phenolic compounds have been reported to possess various pharmacological actions, including antiobesity and antidiabetic actions.1 These compounds exert their effects through different mechanisms such as the reduction of oxidative stress and inflammatory processes, the improvement of glucose and lipid metabolism as well as sensitivity to insulin.2–3 Biological activities of phenolic compounds are known to be strongly dependent on their bioavailability, which is defined as the proportion of the nutrient that is digested, absorbed, and metabolized through normal pathways. Bioavailability differs greatly between the compounds, so that the ones most abundant in the diet are not necessarily those leading to the highest concentrations of metabolites circulating in the plasma. Moreover, the metabolites appearing in the circulation may not have the same bioactivity as that of parent compounds, often determined in vitro.

The general metabolism of phenolic compounds is rather well understood.1,5 During food ingestion, phenolic compounds can be released from the food matrix by mastication and can be hydrolyzed in part by oral microbiota.6 In the stomach, most of the phenolic compounds probably resist acid hydrolysis and arrive intact to the intestine, their major site of absorption.7 Mostly present as esters, glycosides, or polymers, phenolic compounds are very poorly absorbed in their native form and must be hydrolyzed. Phenolic glycosides are deglycosylated in the lumen by membrane-bound lactase-phlorhizin-hydrolase (LPH), and the released aglycones enter the epithelial cells by passive diffusion as a result of an increased lipophilicity.8 The phenolic glycosides may also be deglycosylated in epithelial cells by cytosolic β-glucosidase after they have been transported through the epithelium by sugar transporters such as SGLT1 (sodium-dependent glucose transporter).9 Once absorbed, the phenolic aglycones undergo methylation, glucuronidation, and sulfation.10 This conjugation process, which occurs mainly in liver, represents a metabolic detoxification process, which aims to facilitate the excretion of phenolic compounds by increasing their
hydrophilicity. Hepatic metabolites can then return to the intestinal lumen via the enterohepatic circulation and undergo further transformations. Then, luminal phenolic metabolites and phenolic compounds that have not been absorbed by the intestine reach the colon where they are subjected to microbial degradation. Microbial metabolites are absorbed from the colon and are also subjected to the conjugation processes in the liver, resulting in their glucuronidated and sulfated derivatives. Finally, the phenolic microbial metabolites are excreted from the body via urine as hepatic conjugates. The absorption, as well as the extent of hepatic conjugation and microbial deconjugation, is strongly affected by the structure of phenolic compounds. Although hepatic metabolism leads to the generation of numerous combinations of methylated, glucuronidated, and/or sulfated phenolic metabolites, colonic microbial metabolism follows a general pattern leading to a relatively small number of metabolites, mainly phenolic acids and derivatives of phenylpropionic and phenylacetic acids.

Direct evidence of the bioavailability of phenolic compounds can be obtained by characterizing the profile of metabolites in the plasma. This requires the use of a sensitive analytical methodology such as UHPLC with tandem MS, associated with microelution SPE (μSPE) technology, which allows the characterization of numerous metabolites in biological fluids at low concentrations, in a small volume of sample and in a short time.

Among foods rich in phenolic compounds, strawberry, cranberry, and onion have previously demonstrated some beneficial effects on blood glucose regulation in rodents, constituting a promising natural therapeutic approach in the prevention of obesity and type-2 diabetes. The purpose of the present work was thus to study the bioavailability of strawberry and cranberry phenolic compounds in a representative context of a diet-induced obesity model (rats fed a high-fat, high-sucrose diet) and compare it to a concomitant supplementation with an onion extract. Male Wistar rats were either administered a vehicle, a single intake of GlucoPhenol extract, or the same intake of onion extract. Blood samples were collected pre- and postgestion, and plasma phenolic metabolites were characterized using an optimized UHPLC-MS/MS method, after their extraction with μSPE, and compared with the native phenolic composition of the studied extracts.

## MATERIALS AND METHODS

### Plant Material

Strawberry-cranberry (Fragaria × ananassa, Duch. cv. authentique, and Vaccinium macrocarpon L) extracts blend GlucoPhenol (GP) and onion (Allium cepa L) extract were provided by Nutra Canada company (total phenolic content of 18% and 45% dry weight respectively, as determined by Folin–Ciocalteu method).

### Chemicals

The following phenolic standards were purchased from Sigma-Aldrich (St. Louis, MO): ellagic acid, 5-cafeoylquinic acid, protocatechuic acid, p-hydroxybenzoic acid, p-coumaric acid, vanillic acid, gallic acid, quercetin, quercetin 3-glucoside, kaempferol, myricetin, isorhamnetin, catechin, epicatechin, and rosmarinic acid. Ascorbic acid, citric acid, and hydrochloric acid were also purchased from Sigma-Aldrich (St. Louis, MO). Pelargonidin 3-glucoside was obtained from Extrasynthese (France). Liquid chromatography grade solvents acetone, methanol, and acetonitrile were purchased from EMD Millipore Chemicals (Billerica, MA), and formic acid, glacial acetic acid, and phosphoric acid were obtained from Anachemia (Canada). Ultrapure water was obtained from a Millipore Milli-Q water purification system (Billerica, MA).

### Determination of Strawberry–Cranberry and Onion Extracts Phenolic Composition

#### Characterization of Anthocyanins

The anthocyanin composition of GP extract was analyzed as previously described by reverse-phase analytical HPLC using an Agilent 1100 series system (Santa Clara, CA). The separation was performed with a flow rate of 1 mL/min using a Develosil C18 reverse phase column (250 mm × 4 mm, 5 μm particle size), protected with an Ultrasep C18 guard column (Phenomenex, CA). A binary gradient of 5% formic acid in ultrapure water (solvent A) and methanol (solvent B) was as follows for the anthocyanins separation: 0–2 min, 5% B; 2–10 min, 5%–20% B; 10–15 min, 20% B; 15–30 min, 20–25% B; 30–35 min, 25% B; 35–50 min, 25–33% B; 50–55 min, 33% B; 55–65 min, 33–36% B; 65–70 min, 36–45% B; 70–75 min, 45–53% B; 75–80 min, 53–55% B; 80–84 min, 55–70% B; 84–88 min, 70–5% B; 88–90 min, 5% B. Chromatographic data were acquired at 520 nm, and the quantification was performed using pelargonidin 3-glucoside as standard.

#### Characterization of Procyanidins

The procyanidin composition of GP extract was analyzed as previously described by normal-phase analytical HPLC using an Agilent 1260/1290 infinity system (Santa Clara, CA) equipped with a fluorescence detector. The separation was performed at 35°C with a flow rate of 0.8 mL/min using a Develosil Diol column (250 mm × 4.6 mm, 5 μm particle size), protected with a Cyano SecurityGuard column (Phenomenex, CA). The elution was performed using a solvent system comprising solvents A (acetonitrile/acetate acid 98/2 v/v) and B (methanol/ultrapure water/acetate acid 95/3/2 v/v/v) mixed using a linear gradient from 0% to 40% B for 35 min, 40% to 100% B for 5 min, 100% isocratic B over 5 min, and 100% to 0% B for 5 min. The fluorescence was monitored at excitation and emission wavelengths of 230 and 321 nm. Procyanidins with degrees of polymerization (DP) from 1 to >10 were quantified using an external calibration curve of epicatechin, taking into account their relative response factors in fluorescence.

#### Determination of Total Ellagitannins

Total ellagitannins were estimated by reverse-phase analytical HPLC quantification of released ellagic acid following acid hydrolysis. Briefly, GP extract was dissolved in 25 mL of 50% methanol in ultrapure water containing ascorbic acid and hydrochloric acid (final concentrations of 10 mM and 1.2 M respectively). The mixture was heated to 85°C in the dark for 2 h and then cooled in ice for 5 min. Ellagic acid separation was performed on an Agilent 1100 series HPLC system (Santa Clara, CA) using a Develosil C18 reverse phase column (250 mm × 4 mm, 5 μm particle size), protected with an Ultrasep C18 guard column (Phenomenex, CA, USA). The elution was performed at a flow rate of 1 mL/min using a solvent system comprising solvents A (methanol/ultrapure water/acetic acid 10/88/2 v/v/v) and B (methanol/ultrapure water/acetic acid 90/8/2 v/v/v) mixed using a gradient as follows: 0–15 min, 0–15% B; 15–25 min, 15–50% B; 25–34 min, 50–70% B; 34–35 min, 70–0% B. Chromatographic data were acquired at 250 nm, and the quantification was performed using ellagic acid standard.

#### Characterization of Phenolic Acids and Flavonoids

Phenolic acids and flavonoids were characterized using a Waters Acuity UPLC-MS/MS equipped with an H-Class quaternary pump system, a flow through needle (FTN) sample manager system, and a column manager. The MS detector was a TQD mass spectrometer equipped with a Z-spray electrospray...
The analysis was achieved using an Agilent Plus C18 column (2.1 mm × 100 mm, 1.8 μm) (Santa Clara, CA). The separation was performed at 40 °C at a flow rate of 0.4 mL/min with a mobile phase consisting of 0.1% formic acid in ultrapure water and acetonitrile (solvent A and B, respectively) using a gradient elution as follows: 0–4.5 min, 5–20% B; 4.5–6.45 min, 20% B; 6.45–13.5 min, 20–45% B; 13.5–16.5 min, 45–100% B; 16.5–19.5 min, 100% B; 19.5–19.52 min, 100–5% B; 19.52–22.5 min, 5% B. The MS/MS analyses were carried out in negative mode using electrospray source parameters as follows: electrospray capillary voltage was 2.5 kV, source temperature was 140 °C, desolvation temperature was 350 °C, and cone and desolvation gas flows were 80 l/h and 900 l/h, respectively. Data were acquired through multiple reaction monitoring (MRM) using Waters Masslynx V4.1 software. Phenolic standards were analyzed using the same parameters and used for the quantification, when available. Otherwise, the phenolic compounds were quantified using their aglycone or the most similar phenolic structure.

Animals and Plasma Preparation. Sixty male Wistar rats (Charles River, St. Constant, QC) were placed in temperature- and humidity-controlled rooms (21 ± 2 °C, 35–40%), with a daily 12h–12h light–dark cycle. Animal facilities met the guidelines of the Canadian Council on Animal Care, and the protocols were approved by the Animal Care Committee of Laval University (reference CPAUL-2011-111). Animals were acclimated to their environment for a minimum of 5 days and consumed a nonpurified rodent diet ad libitum (rodent chow no. 2918, Harlan Teklad, Madison, WI). All animals had continuous access to tap water. After the acclimation period, rats were fed a high-fat, high-sucrose (HFHS) diet containing 27% sucrose and 40% fat for 7–8 days. The day before the test, rats were fasted for 12 h. The animals were randomly allocated to 6 groups (9 to 12 per group, Table 1). A first control group (group 1) consisted of animals which only received the vehicle (0.1% citric acid in water). The animals of four groups (groups 2–5) were administered a single dose of GP extract corresponding to a phenol intake of 2.7, 5.4, 27, and 36 mg/kg, respectively. The animals of an additional experimental group (group 6) were administered a single dose of GP extract supplemented with onion extract, corresponding to a total phenol intake of 36 mg/kg (5.4 mg/kg from GP extract and 30.6 mg/kg from onion extract). Blood samples were collected from the saphenous vein with EDTA-containing syringes preingestion and at 30, 60, 120, 180, and 360 min postingestion. Plasma samples were obtained by centrifugation (3500 rpm, 10 min at 4 °C) and stored at −80 °C until analysis.

### Table 1. Description of Phenolic Supplementation Treatments: Dose of Extract, Equivalent Phenolic Content, and Total Phenolic Intake (mg/kg of Weight Body)

<table>
<thead>
<tr>
<th>Group Identification</th>
<th>Dose of Extract (mg/kg)</th>
<th>Dose of Phenolic Compounds (mg/kg)</th>
<th>Total Phenolic Intake (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2) 2.7 mg/kg GP</td>
<td>15</td>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td>(3) 5.4 mg/kg GP</td>
<td>30</td>
<td>5.4</td>
<td>5.4</td>
</tr>
<tr>
<td>(4) 27 mg/kg GP</td>
<td>150</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>(5) 36 mg/kg GP</td>
<td>200</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>(6) 36 mg/kg GP + O</td>
<td>30</td>
<td>68</td>
<td>5.4</td>
</tr>
</tbody>
</table>

*The experimental groups are numbered from (1) to (6).*

Extraction of Plasma Phenolic Compounds. Phenolic compounds extraction was realized according to a previously described methodology. Waters Oasis HLB μelution plates (Milford, MA) were preconditioned using 250 μL of methanol and 250 μL of 0.2% acetic acid. Plasma samples (20 to 200 μL) were mixed with 4% phosphoric acid in ultrapure water (v/v) to disrupt phenol–protein binding prior loading into the plates. The loaded plates were washed with 200 μL of ultrapure water and 200 μL 0.2% acetic acid, and the retained phenolic compounds were then eluted with 2 × 50 μL of acetonitrile/ultrapure water/acetic acid solution (AWA) 70/29.5/0.5 v/v/v. Rosmarinic acid (1 μg/mL final concentration) was added in AWA elution solution and used as internal standard to check the homogeneity of the SPE extraction procedure. The eluted solutions, in the collecting plates, were directly injected in UHPLC-MS/MS for phenolic acids and nonanthocyanins flavonoids analysis (negative mode). The extracts were then lyophilized and dissolved in 30 μL of 20% methanol 0.1% acetic acid for anthocyanins analysis (positive mode).

Characterization of Metabolites by UHPLC-MS. The analysis of plasma extracted phenolic metabolites was achieved with UHPLC-MS/MS using a previously developed methodology for the detection of anthocyanins and procyanidins, slightly modified to also allow the detection of phenolic acids and flavonols.

The separation of phenolic acids and nonanthocyanins flavonoids was performed at 30 °C using an Agilent Plus C18 column (2.1 mm × 100 mm, 1.8 μm) (Santa Clara, CA). A flow rate of 0.4 mL/min was used with a mobile phase consisting of 0.2% acetic acid in ultrapure water and acetonitrile (solvent A and B, respectively) following this gradient elution: 0–8 min, 5–50%; 8–9.10 min, 50–90%; 9.10–10 min, 90% B; 10–10.10 min, 90–5%; 10.10–13 min, 5% B. The MS/MS analyses were carried out in negative mode using electrospray source parameters as follows: electrospray capillary voltage was 3.01 kV, source temperature was 150 °C, desolvation temperature was 400 °C, and cone and desolvation gas flows were 800 l/h and 800 l/h, respectively.

The separation of anthocyanins was performed at 30 °C with a flow rate of 0.45 mL/min with a mobile phase consisting of 10% acetic acid in ultrapure water and acetonitrile (solvent A and B, respectively) using a gradient elution as follows: 0–3.50 min, 2–50%; 3.50–4.5 min, 50–90%; 4–4.50 min, 90% B; 4.50–4.55 min, 90–2% B; 4.55–6 min, 2% B. The MS/MS analyses were carried out in positive mode using electrospray source parameters as follows: electrospray capillary voltage was 1.3 kV, source temperature was 130 °C, desolvation temperature was 350 °C, and cone and desolvation gas flows were 800 l/h and 900 l/h, respectively.

Cone voltage and collision energy parameters were optimized for each compound. The identification of metabolites was performed by comparing their retention times with those of available phenolic standards and/or analyzing their fragmenta-
RESULTS

Phenolic Composition of Strawberry–Cranberry and Onion Extracts. The phenolic composition of the two extracts is presented in Table 3. GP extract contained 0.8% of anthocyanins, with the main presence of pelargonidin 3-glucoside. This extract also contained about 3% of total procyanidins with a large spectrum of degree of polymerization, the monomers catechin and epicatechin representing around 0.1%. The extract presented a total ellagitannins content of 1.8%, including 0.8% of free ellagic acid. About 1.3% of phenolic acids were also identified in this extract, mainly chlorogenic acid and p-coumaric acid, and 1.3% of flavonoids with the main presence of quercetin 3-glucoside. Conversely, onion extract did not contain any anthocyanins, procyanidins, or ellagitannins and contained almost exclusively aglycone quercetin and protocatechuic acid (about 14% and 8%, respectively). Chromatographic profiles of anthocyanidins and proanthocyanidins of GP extract are available as Supplemental Figures S1 and S2, respectively.

Rat Plasma Phenolic Content. The evolution of the total phenolic concentration in rat plasma after ingestion of the extracts is presented in Figure 1A. The presence of phenolic compounds was detected in all treated groups, with a maximum phenolic concentration ranging from 0.5 to 8 mg/L in GP groups (groups 2–5; phenolic intake from 2.7 to 36 mg/kg) and 10 mg/L for the onion supplemented GP group (group 6; phenolic intake of 36 mg/kg). The kinetic profile of plasma total phenolic metabolites strongly differed depending on the phenolic source. GP-fed animals presented a high phenolic content 30 min after ingestion of the extract, followed by a rapid elimination of the metabolites by the organism (>85% over 360 min). GP/onion fed animals also presented a high phenolic content 30 min after ingestion of the mix, but the metabolites concentration remained high over time. Interestingly, the groups 5 and 6, which have ingested both the same dose of phenolic compounds (36 mg/kg) but from different sources, had the same phenolic concentration 30 min postingestion (about 8 mg/L; p = 0.7829) but a 10-fold

Table 2. Phenolic Standards, MRM Transitions, and Optimized MS Parameters Used for the Analysis of Native Phenolic Compounds and Their Metabolites by UHPLC-MS

<table>
<thead>
<tr>
<th>compound</th>
<th>standard for quantification</th>
<th>ionization mode</th>
<th>MRM cone voltage (V)</th>
<th>collision energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>phenolic acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-hydroxybenzoic acid</td>
<td>p-hydroxybenzoic acid</td>
<td>negative</td>
<td>137 &gt; 93</td>
<td>35</td>
</tr>
<tr>
<td>p-hydroxybenzoic acid</td>
<td>p-hydroxybenzoic acid</td>
<td>negative</td>
<td>329 &gt; 153</td>
<td>40</td>
</tr>
<tr>
<td>p-hydroxybenzoic acid</td>
<td>p-hydroxybenzoic acid</td>
<td>negative</td>
<td>329 &gt; 153</td>
<td>40</td>
</tr>
<tr>
<td>dimethyl ellagic acid glucuronide</td>
<td></td>
<td>negative</td>
<td>505 &gt; 329</td>
<td>40</td>
</tr>
<tr>
<td>proanthocyanidin</td>
<td>proanthocyanidin</td>
<td>negative</td>
<td>153 &gt; 109</td>
<td>29</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>p-coumaric acid</td>
<td>negative</td>
<td>163 &gt; 119</td>
<td>35</td>
</tr>
<tr>
<td>flavon-3-ols</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>catechin - epicatechin</td>
<td>epicatechin</td>
<td>negative</td>
<td>289 &gt; 245</td>
<td>42</td>
</tr>
<tr>
<td>catechins glucuronide</td>
<td>epicatechin</td>
<td>negative</td>
<td>465 &gt; 289</td>
<td>40</td>
</tr>
<tr>
<td>methyl catechins glucuronide</td>
<td>epicatechin</td>
<td>negative</td>
<td>479 &gt; 303</td>
<td>40</td>
</tr>
<tr>
<td>methyl catechins sulfate</td>
<td>epicatechin</td>
<td>negative</td>
<td>383 &gt; 303</td>
<td>45</td>
</tr>
<tr>
<td>flavonols</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>quercetin</td>
<td>quercetin</td>
<td>negative</td>
<td>301 &gt; 150</td>
<td>42</td>
</tr>
<tr>
<td>quercetin glucuronide</td>
<td>quercetin 3-glucoside</td>
<td>negative</td>
<td>477 &gt; 301</td>
<td>40</td>
</tr>
<tr>
<td>quercetin diglucuronide</td>
<td>quercetin 3-glucoside</td>
<td>negative</td>
<td>653 &gt; 301</td>
<td>40</td>
</tr>
<tr>
<td>quercetin sulfate</td>
<td>quercetin 3-glucoside</td>
<td>negative</td>
<td>381 &gt; 301</td>
<td>40</td>
</tr>
<tr>
<td>isorhamnetin glucuronide</td>
<td>isorhamnetin 3-glucoside</td>
<td>negative</td>
<td>491 &gt; 315</td>
<td>40</td>
</tr>
<tr>
<td>isorhamnetin diglucuronide</td>
<td>isorhamnetin 3-glucoside</td>
<td>negative</td>
<td>667 &gt; 315</td>
<td>40</td>
</tr>
<tr>
<td>isorhamnetin sulfate</td>
<td>isorhamnetin 3-glucoside</td>
<td>negative</td>
<td>395 &gt; 315</td>
<td>40</td>
</tr>
<tr>
<td>isorhamnetin glucuronide sulfate</td>
<td>isorhamnetin 3-glucoside</td>
<td>negative</td>
<td>571 &gt; 315</td>
<td>40</td>
</tr>
<tr>
<td>myricetin glucuronide</td>
<td>myricetin 3-glucoside</td>
<td>negative</td>
<td>493 &gt; 317</td>
<td>40</td>
</tr>
<tr>
<td>myricetin diglucuronide</td>
<td>myricetin 3-glucoside</td>
<td>negative</td>
<td>669 &gt; 317</td>
<td>40</td>
</tr>
</tbody>
</table>

Data Analysis. All phenolic characterizations were carried out in triplicate, and results were expressed as percentage of extract weight ± standard deviation (SD). Time 0 values being very close to 0, phenolic concentrations in rat plasma were expressed as variation of concentration from baseline (Δ mg/L for total phenolic content and Δ nM or Δ μM for individual phenolic compounds). Metabolites quantification was expressed as area under the plasma concentration (μM) time (min) curve (AUC), with AUC calculated according to the linear trapezoidal rule. Results were expressed as means ± standard error (SEM) of the mean. The main effect of phenol intake on AUC values was estimated by one-way analysis of variance (ANOVA) using the MIXED procedure in SAS 9.3 (Cary, NC). When necessary, log transformation was applied on raw data to meet the criteria for normality. A priori contrasts between selected groups were analyzed using the LSD method. The REG procedure of SAS was used to analyze if plasma metabolite concentration (determined as AUC) followed a linear regression model with GP fed animals also presented a high phenolic content 30 min after ingestion of the mix, but the metabolites concentration remained high over time. Interestingly, the groups 5 and 6, which have ingested both the same dose of phenolic compounds (36 mg/kg) but from different sources, had the same phenolic concentration 30 min postingestion (about 8 mg/L; p = 0.7829) but a 10-fold

dx.doi.org/10.1021/jf404965z | J. Agric. Food Chem. XXXX, XXX, XXX–XXX
difference in the phenolic concentration 360 min postingestion (about 1 mg/L for the group 5 and about 10 mg/L for the group 6; p < 0.0001). On the basis of mean rat body weight and blood volume (400 g and 26 mL respectively), absorption rates were estimated up to 1.4% of the total phenolic intake for GP fed rats and up to 1.8% for GP/onion fed rats.

**Plasma Phenolic Metabolites: Identification, Quantification, and Kinetic Profile.** The designed UHPLC-MS methods enabled the characterization of 21 phenolic compounds in rat plasma after the consumption of the extracts. These metabolites are listed in Tables 4 and 5 and their kinetic profiles are represented in Figures 1A−C.

Among anthocyanins, strictly present in GP extract, pelargonidin 3-glucoside and peonidin 3-galactoside were detected in their native form but at an extremely low level (0.01 and 0.001 nM max, respectively). A well-known metabolite

---

**Table 3. Phenolic Composition of GlucoPhenol and Onion Extracts**

<table>
<thead>
<tr>
<th>content (% dry weight)</th>
<th>GlucoPhenol extract</th>
<th>onion extract</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>total phenolic content</strong></td>
<td>18</td>
<td>45</td>
</tr>
<tr>
<td><strong>phenolic acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ellagic</td>
<td>1.32 ± 0.04</td>
<td>8.49 ± 0.33</td>
</tr>
<tr>
<td>chlorogenic acid</td>
<td>0.78 ± 0.00</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td>protocatechuic acid</td>
<td>0.16 ± 0.01</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>p-hydroxybenzoic acid</td>
<td>0.04 ± 0.00</td>
<td>8.39 ± 0.04</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>&lt;0.01</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td>vanillic acid</td>
<td>0.14 ± 0.00</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>caffeoyl glucoside</td>
<td>0.03 ± 0.01</td>
<td>0.04 ± 0.00</td>
</tr>
<tr>
<td>feruloyl glucoside</td>
<td>0.05 ± 0.00</td>
<td>-</td>
</tr>
<tr>
<td>coumaroyl glucoside</td>
<td>0.06 ± 0.00</td>
<td>-</td>
</tr>
<tr>
<td><strong>flavonoids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>flavonols</td>
<td></td>
<td></td>
</tr>
<tr>
<td>quercetin</td>
<td>2.26 ± 0.02</td>
<td>14.78 ± 0.27</td>
</tr>
<tr>
<td>quercetin 3-glucoside</td>
<td>1.35 ± 0.02</td>
<td>14.78 ± 0.27</td>
</tr>
<tr>
<td>quercetin diglucoside</td>
<td></td>
<td></td>
</tr>
<tr>
<td>quercetin galactoside</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>quercetin rhamnoside</td>
<td>0.02 ± 0.00</td>
<td>0.05 ± 0.00</td>
</tr>
<tr>
<td>quercetin xyloside</td>
<td>0.16 ± 0.00</td>
<td>-</td>
</tr>
<tr>
<td>quercetin arabinoside</td>
<td>0.06 ± 0.00</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>kaempferol</td>
<td>0.00 ± 0.01</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td>kaempferol glucoside/galactoside</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>myricetin</td>
<td>0.15 ± 0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>myricetin glucoside/galactoside</td>
<td>0.17 ± 0.00</td>
<td>-</td>
</tr>
<tr>
<td>isorhamnetin</td>
<td>0.02 ± 0.00</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td><strong>total anthocyanins</strong></td>
<td>0.80 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>cyanidin 3-galactoside</td>
<td>0.10 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>cyanidin 3-glucoside</td>
<td>0.02 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>cyanidin 3-arabinoside</td>
<td>0.09 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>pelargonidin 3-glucoside</td>
<td>0.35 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>peonidin 3-galactoside</td>
<td>0.12 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>peonidin 3-glucoside</td>
<td>0.01 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>peonidin 3-arabinoside</td>
<td>0.07 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>pelargonidin 3-(malonoyl)-glucoside</td>
<td>0.04 ± 0.00</td>
<td></td>
</tr>
<tr>
<td><strong>flavan-3-ols</strong></td>
<td>0.11 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>catechin</td>
<td>0.05 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>epicatechin</td>
<td>0.06 ± 0.01</td>
<td></td>
</tr>
<tr>
<td><strong>total procyanidins</strong></td>
<td>3.24 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>monomers</td>
<td>0.32 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>dimers</td>
<td>0.98 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>trimers</td>
<td>0.46 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>tetramers</td>
<td>0.32 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>pentamers</td>
<td>0.17 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>hexamers</td>
<td>0.13 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>heptamers</td>
<td>0.06 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>octamers</td>
<td>0.03 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>nonamers</td>
<td>0.02 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>polymers (DP &gt; 10)</td>
<td>0.75 ± 0.02</td>
<td></td>
</tr>
<tr>
<td><strong>total ellagitans</strong></td>
<td>1.77 ± 0.07</td>
<td></td>
</tr>
</tbody>
</table>

“Results are expressed as mean of triplicate ± SD. "-" Not detected.”
Figure 1. continued
Figure 1. continued
of pelargonidin 3-glucoside, p-hydroxybenzoic acid, was detected in significant amounts (12 μM max) and was also detected in its glucuronidated form, in a 10 times lower quantity. Among flavan-3-ols, also strictly present in GP extract, catechin and epicatechin were not detected in significant amounts in their native form but under conjugated forms. As the conjugated forms of catechin and epicatechin were not distinguishable by MS, these isomers were quantified together and referenced as “catechins”. Catechins metabolites were detected under glucuronidated and both methylated and glucuronidated forms (until 20 μM max). A

Figure 1. Evolution of the total phenolic concentration in rat plasma after ingestion of GlucoPhenol extract with or without onion extract. Results are expressed as mean of replicate (9 to 12) ± SEM. GP: GlucoPhenol; GP + O: GlucoPhenol + onion.
were also detected (0.1 \text{ low amount of both methylated and sulfated forms of catechins}). Protocatechuic acid, a phenolic acid present in a very high amount in onion extract and in a low amount in GP extract, was detected in its native aglycone form in a very low amount (0.2 \mu M max). Among phenolic acids, p-coumaric acid and a well-known degradation product of the ellagitannins, dimethyl ellagic acid glucuronide, were detected in significant amounts (until 10 \mu M). Native anthocyanidins, metabolites of flavan-3-ols and p-coumaric acid, presented a kinetic profile with a high concentration 30–60 min postingestion followed by a progressive decrease, whereas dimethyl ellagic acid glucuronide showed an increase of its concentration until 180 min post-ingestion followed by a plateau until 360 min post-ingestion.

Protocatechuic acid, a phenolic acid present in a very high amount in onion extract and in a low amount in GP extract, was detected in high quantities (15 \mu M max) and presented a kinetic profile similar to that of p-coumaric acid with a concentration peak at 30 min post-ingestion. Flavonols were the major phenolic constituents of onion extract, but they were also present in GP extract in smaller quantities. Quercetin, detected in its native aglycone form in a very low amount (0.2 \mu M max), was mostly detected in circulation in its conjugated forms: glucuronidated, diglucuronidated, and sulfated quercetin (30, 45, and 1.5 \mu M max, respectively); it was also detected as conjugates of its methylated form isorhamnetin: glucuronidated, diglucuronidated, and sulfated isorhamnetin (about 15–20 \mu M max). A very high concentration of both a glucuronidated and a sulfated form of isorhamnetin was also detected (about 120 \mu M max). Some metabolites of myricetin, present in very low quantities in GP extract, were also detected under glucuronidated and diglucuronidated forms (15 and 6 \mu M max, respectively). The kinetic profiles of the glucuronidated forms of quercetin were characterized by an increase of its concentration until 60 min post-ingestion followed by a plateau until 360 min post-ingestion, whereas the sulfated form of quercetin showed a continuous increase until 360 min post-ingestion. All the isorhamnetin metabolites also displayed a kinetic profile with a strong increase of their concentration until 360 min post-ingestion. Glucuronidated myricetin reached a high concentration peak 30 min post-ingestion followed by a rapid decline, whereas diglucuronidated myricetin reached its highest concentration 120 min post-ingestion and remained constant until 360 min post-ingestion.

**Statistical Analysis: Linear Regression and A Priori Comparison between Groups.** The individual metabolites, together with their AUC values and their statistical parameters, are listed in Tables 4 and 5. A linear regression analysis was performed on individual metabolite concentrations (expressed as AUC values) quantified in control and GP groups (groups 1 to 5) and GP ingested dose (phenolic intake from 0 mg/kg to 36 mg/kg). Almost all the detected phenolic metabolites fitted with the linear regression model (R^2 ranging from 0.267 to 0.775), with the exception of native catechins and quercetin (p = 0.5161 and 0.7592, respectively). These results indicate that, in the range of tested doses, there is a relatively strong proportionality between the amount of phenolic compounds...
ingested by animals and the concentration of phenolic metabolites in plasma.

A comparison was made between the groups 5 and 6 for the total circulating phenolic concentration, expressed in AUC values (Table 4). Whereas the rats of these two groups have ingested the same phenolic dose (36 mg/kg), the total AUC value in group 6 was more than 3-fold the AUC value in group 5 (p < 0.0001). The groups 3 and 6 were also compared for their individual circulating metabolites concentrations, expressed in AUC values (Tables 4 and 5). These two groups have ingested the same GP dose (5.4 mg/kg), but the animals of group 6 have also received an onion supplementation, resulting in a different total phenolic intake (5.4 mg/kg and 36 mg/kg for groups 3 and 6, respectively). Protocatechuic acid and flavonol metabolites, mainly coming from onion extract, obviously presented strongly different AUC values between these two groups (from 5- to 329-fold more in group 6; p ≤ 0.0005) as only the animals of group 6 have received the onion extract (Table 5). Among phenolic metabolites coming from GP extract (Table 4),peonidin 3-galactoside, pelargonidin 3-glucoside, p-hydroxybenzoic acid, p-coumaric acid, and methyl catechins sulfate presented as expected close AUC values in groups 3 and 6 (p value ranging from 0.1502 to 0.9467) as all the animals have received the same GP dose. However, glucuronlated metabolites showed higher AUC values in group 6 than in group 3, meaning that for the same intake of GP extract, the onion-supplemented animals have produced more glucuronide metabolites of strawberry–cranberry phenolic compounds. Indeed, p-hydroxybenzoic glucuronide, catechins glucuronide, and methyl catechins glucuronide AUC values were found higher in group 6 in comparison with group 3: +252% (p = 0.0001), +279% (p = 0.0018), and +118% (p = 0.0131), respectively. The glucuronidated dimethyl ellagic acid was an exception, because it showed similar AUC values in the two groups (p = 0.8352). UHPLC-MS/MS MRM profiles of p-hydroxybenzoic glucuronide, catechins glucuronide, and methyl catechins glucuronide are available as Supplemental Figure S3.

### Discussion

The present study aimed at investigating the bioavailability of strawberry and cranberry phenolic compounds after oral administration to rats, with or without onion supplementation. The human daily intake of plant phenols is estimated to be between 150 and 1000 mg.\(^{20}\) In this study, the rats received a phenol intake from 2.7 to 36 mg/kg, which corresponds to a human intake from 25 to 333 mg (mean body weight of 60 kg) according to the Food and Drug Administration’s (FDA) body surface area (BSA) normalization.\(^{21}\) The selected doses were lower than the recommended doses in order to be relevant in a nutritional supplementation context and to be further compared with human clinical trials.

The evidence of the absorption of phenolic compounds was earlier based on the increase of the antioxidant status of the plasma after the consumption of phenolic-rich foods. This

---

**Table 5. Concentration of Onion Phenolic Metabolites (Expressed as AUC) Found in the Plasma of Rats Fed with Different Concentrations of GlucoPhenol Extract, Co-Ingested or Not with Onion Extract**

<table>
<thead>
<tr>
<th></th>
<th>quercetin glucuronide</th>
<th>quercetin diglucuronide</th>
<th>quercetin sulfate</th>
<th>isorhamnetin glucuronide</th>
<th>isorhamnetin diglucuronide</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AUC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) control</td>
<td>11.3 ± 3.9</td>
<td>15.8 ± 3.8</td>
<td>7.0 ± 2.2</td>
<td>4.2 ± 1.4</td>
<td>12.8 ± 3.2</td>
</tr>
<tr>
<td>(2) 2.7 mg/kg GP</td>
<td>10.5 ± 6.2</td>
<td>22.0 ± 4.0</td>
<td>55.0 ± 6.6*</td>
<td>3.2 ± 1.2</td>
<td>59.1 ± 13.1*</td>
</tr>
<tr>
<td>(3) 5.4 mg/kg GP</td>
<td>7.4 ± 4.6</td>
<td>32.6 ± 10.0</td>
<td>83.5 ± 22.3*</td>
<td>10.2 ± 8.5</td>
<td>69.1 ± 14.6*</td>
</tr>
<tr>
<td>(4) 27 mg/kg GP</td>
<td>9.2 ± 2.3</td>
<td>240 ± 37*</td>
<td>612 ± 94*</td>
<td>17.2 ± 4.8*</td>
<td>605 ± 96*</td>
</tr>
<tr>
<td>(5) 36 mg/kg GP</td>
<td>8.9 ± 2.2</td>
<td>369 ± 63*</td>
<td>814 ± 111*</td>
<td>37.3 ± 10.7*</td>
<td>852 ± 125*</td>
</tr>
<tr>
<td>(6) 36 mg/kg GP + O</td>
<td>37.1 ± 8.8*</td>
<td>8629 ± 1157*</td>
<td>13290 ± 1529*</td>
<td>304 ± 77*</td>
<td>6481 ± 693*</td>
</tr>
</tbody>
</table>

**Statistics**

- **Linear regression**
  - quercetin glucuronide: \( p = 0.7592 \) (\( R^2 = 0.002 \))
  - quercetin diglucuronide: \( p = 0.0001 \) (\( R^2 = 0.0655 \))
  - quercetin sulfate: \( p = 0.0001 \) (\( R^2 = 0.740 \))
  - isorhamnetin glucuronide: \( p = 0.0001 \) (\( R^2 = 0.267 \))
  - isorhamnetin diglucuronide: \( p = 0.0001 \) (\( R^2 = 0.716 \))

- **A priori contrasts**
  - (3) vs (6): \( p = 0.0005 \)
  - (5) vs (6): \( p = 0.0015 \)

**Results are expressed as mean of replicate (9 to 12) ± SEM. GP: GlucoPhenol; GP + O: GlucoPhenol + onion. The experimental groups are numbered from (1) to (6). \(^{p} < 0.05\) in comparison with control group.**

---

\( \text{dx.doi.org/10.1021/jf404965z} \)
approach has been largely criticized, and direct measurements of phenolics in plasma using HPLC with DAD or MS detection have been preferred but provided limited information due to a low sensitivity. Numerous bioavailability studies were conducted using enzymatic hydrolysis but provided limited information regarding the actual metabolites present in biological fluids. To obtain an overview of the absorption and metabolism of phenolic compounds, it is essential to be able to characterize most of the circulating metabolites, generally present at low concentrations. This requires the use of an appropriate extraction method to isolate phenolic metabolites from plasma-like microelution SPE (μSPE) and a sensitive analytical methodology such as UHPLC with tandem MS for their detection and quantification. The μSPE plates are packed with a high-capacity sorbent both lipophilic and hydrophilic to give maximum retention ability for different polarity compounds from a biological matrix. In comparison with conventional SPE cartridges, μSPE plates present a better extraction recovery and a better preservation of the molecular integrity. Indeed, the eluate can directly be analyzed in the collecting plates in UHPLC-MS, and thus an evaporation step previous to the chromatographic analysis can be avoided. The optimized methods developed allowed the characterization of 21 phenolic metabolites in a volume as small as 20 μL of rat plasma after their consumption of GP extract supplemented or not with onion extract.

As expected when using nutritional doses of phenolic compounds, the phenolic compound transporters and metabolic pathways were probably not saturated, which could explain the general linear increase in the concentration of plasma phenolic metabolites in response to the increase in the dose ingested of GP extract. After ingestion of the extracts, the native phenolic compounds were extensively metabolized and were thus present in the circulation almost exclusively as conjugate metabolites and microbial degradation products.

Quercetin (both aglycone and glycosides forms) was intensively conjugated in glucuronidated and diglucuronidated forms, and to a lesser extent in sulfated form. Quercetin was especially methylated at a high level into isorhamnetin, leading to the formation of isorhamnetin metabolites being present in high quantities, whereas the extracts contained only low levels of isorhamnetin. Isoflavone glucuronide sulfates was the metabolite identified in the highest quantity in rat plasma following the extracts ingestion, showing the intensive metabolism of quercetin, with both methyl, glucuronide, and sulfate conjugations. Moreover, the presence of myricetin metabolites in onion-fed rats’ plasma, even though the onion extract did not contained any myricetin, suggests a probable hydroxylation of quercetin during its metabolism. A wide variety of metabolites resulting from quercetin conjugation has been previously reported in rats and humans. Similarly, catechins monomers have undergone extensive conjugation processes with high contents of glucuronidated forms of catechins and methylated catechins, as well as low content of sulfated forms of methylated catechins detected in strawberry—cranberry-fed animals. Such catechins conjugates were earlier reported in rat plasma.

Anthocyanins constitute an exception to the classical deconjugation—reconjugation sequence involved in the metabolism of phenolic compounds as they can be absorbed in their native glycoside structure. These compounds are known to be poorly available due to their microbial and spontaneous degradation and also to their high sensitivity to pH. Pelargonidin 3-glucoside and peonidin 3-galactoside were thus detected in circulation but in extremely low concentrations. However, high amounts of a known degradation product of pelargonidin 3-glucoside, p-hydroxybenzoic acid, and its glucuronidated conjugate were identified in rat plasma following GP extract consumption.

As expected, no polymeric phenolic compounds were detected in rat plasma. These compounds, such as procyanidins and ellagitannins, are known to be poorly absorbed. Ellagitannins are depolymerized in stomach followed by the absorption of ellagic acid directly in the stomach or in the intestine. Ellagic acid metabolization then leads to the formation of ellagic acid derivatives, like the dimethyl ellagic acid glucuronide detected here, and urolithins. Procyanidins reach the colon where they are subjected to microbial degradation leading to the generation of phenolic acids, which are further absorbed and metabolized. Some phenolic acids were detected here, either coming from the release from food matrix or resulting from more complex phenolic structure degradation. Protocatechuic acid, which was present in GP extract in the same amount as chlorogenic acid, was detected in high quantities in rat plasma following their ingestion of this extract, whereas chlorogenic acid was not detected at all. Chlorogenic acid is poorly metabolized because of a lack of esterases in the intestine tissues. Consequently, the only significant site for chlorogenic acid metabolism is the colonic microflora, which generates a wide variety of metabolites, including p-coumaric acid. 

Identified phenolic metabolites presented some specific kinetic profiles, reflecting the biological site of conjugation. Indeed, a low $T_{\text{max}}$ may be indicative of conjugation taking place in the intestine before the prior passage of metabolites in the circulation, whereas a higher $T_{\text{max}}$ may reflect a postabsorption conjugation. Therefore, glucuronidated catechins conjugates, which presented a high concentration 30–60 min postingestion followed by a progressive decrease, appeared to be early conjugated in the intestine. The delay in the elimination time of methyl catechins glucuronide metabolite suggested a postabsorption methylation of glucuronidated catechins in the liver.

Similarly, glucuronidated quercetin seemed to be conjugated early in the intestine and accumulate over time, which has previously been demonstrated in humans and has been attributed to the high affinity of this compound with serum albumin. The increasing plasma concentration of sulfated quercetin over time probably reflects its hepatic formation. Regarding the kinetic profiles of isorhamnetin metabolites, it appears that they were likely resulting from a postabsorption methylation of already conjugated quercetin metabolites, in the liver.

Anthocyanins are highly soluble and among the few compounds that can be directly absorbed from the stomach, leading to their probable rapid intestinal metabolism, as reflected by their kinetic profiles and those of their metabolites. The kinetic profile of dimethyl ellagic acid glucuronide, a biomarker of the ellagitannins intake, suggests an extensive hepatic metabolization and active enterohepatic circulation of this compound. However, the solubility of phenolic compounds should be a factor to consider as it can greatly influence the kinetics of metabolites. Indeed, because quercetin and ellagic acid
are poorly soluble, their metabolites might precipitate in blood and thus be delayed in their circulation and metabolization.

The absorption and metabolism of phenolic compounds was shown to be strongly modulated by the structure of the phenolic compounds. Indeed, although a similar phenolic metabolites absorption rate was observed 30 min post ingestion in groups 5 and 6 (Figure 1A—total metabolites), which have ingested both the same phenolic amount but from different sources (GP extract or GP supplemented with onion extract), a significantly different absorption rate was observed 360 min post digestion, with the elimination of strawberry—cranberry phenolic metabolites and the accumulation of onion metabolites in rat plasma. These results support the idea that the phenolic structure prevails over the phenolic dose for absorption and metabolization. In all cases, and in accordance with previous reported data, the estimated phenolic absorption was low, as it did not exceed 2% of the ingested phenolic dose. Despite the low circulating concentrations of phenolic metabolites and their rapid elimination (for most of them), those compounds exert various regulating biological activities in vivo, which go beyond direct antioxidant activity.40

Phenolic compound absorption and metabolism can be modulated by phenolics themselves. Indeed, some phenolic compounds are known to specifically modify some metabolic processes that govern bioavailability, such as the modulation of gut microbiota or xenobiotics transporters activity, as well as the modulation of conjugation enzymes.41

Here, we observed the presence of four glucuronidated metabolites resulting from strawberry—cranberry extract phenolic compounds. Among them, three compounds, p-hydroxybenzoic acid glucuronide, catechins glucuronide, and methyl catechins glucuronide, which were suggested to be produced in the intestine regarding their kinetic profile, were found in 2 to almost 4 times higher concentrations in the plasma of animals that have ingested onion extract together with GP extract, for the same GP intake. On the contrary, dimethyl ellagic acid glucuronide, a highly metabolized compound whose kinetic profile indicated a hepatic conjugation, were present in the same quantities in the animals of the two groups. Glucuronidation processes of some strawberry—cranberry phenolic metabolites thus appear to be enhanced in the presence of onion phenolic compounds but specifically in the intestine. This modulation could be attributed to quercetin, which has already been demonstrated to be involved in the induction of glucuronidation enzyme UDP-glucuronosyl-transferase in intestinal cell lines in vitro and in rats in vivo.42–44 These data therefore indicate an opportunity to increase the circulating metabolites concentration using synergistic interactions between phenolic compounds and thus to increase the bioactivity of some plant extracts by improving their oral bioavailability.

In conclusion, this study reported the use of an optimized technique combining μSPE and UHPLC-MS/MS methods for the simultaneous identification and quantification, for the first time, of about 20 phenolic metabolites in rat plasma following the ingestion of GP, a strawberry—cranberry extracts blend alone or with an onion extract. In most cases, native compounds were not detected in rat plasma and were extensively metabolized into conjugates and degradation products. Quercetin metabolites were found in greater amounts in plasma, the rats being supplemented with the onion extract or not, illustrating the differences existing between phenolic compounds in terms of absorption and metabolization. This study also highlighted a possible synergistic activity of phenolic compounds for improving bioavailability, with a possible induction of intestinal UDP-glucuronosyl-transferase by onion quercetin. This work allowed the identification and quantification of actual metabolites generated in vivo following phenolic consumption, as well as the analysis of their pharmacokinetic, data which are fundamental to unravel their mechanisms of action.

■ ASSOCIATED CONTENT

▲ Supporting Information

HPLC chromatograms of GlucoPhenol extract anthocyanins and procyanidins (Figure S1 and Figure S2, respectively) and MRM signals of p-hydroxybenzoic acid glucuronide, catechins glucuronide, and methyl catechins glucuronide in rat plasma (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

E-mail: Yves.Desjardins@sfaa.ulaval.ca. Fax: (1) 418-656-3515. Tel.: (1) 418-656-2131 #2359.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Dr. André Gosselin and Sébastien Léonhart from Nutra Canada and Dr. Barry Ritz from Atrium Innovations for supplying the experimental extracts and critically reviewing the manuscript. Financial support was provided by the Quebec Industrial Bioprocess Research Consortium (CRIBIQ) of the Ministry of Economic Development, Innovation, and Export Trade (MDEIE), Nutra Canada, and Atrium Innovations.

■ ABBREVIATIONS:

SPE: solid phase extraction; DP: degree of polymerization; GP: GlucoPhenol; O: onion; \( T_{\text{max}} \): time to reach maximum concentration; AWA: acetone—water—acetic acid

■ REFERENCES


(22) Lotito, S. B.; Frei, B. Consumption of flavonoid-rich foods and cardiovascular health in humans is not established. FEBS Lett. 2009, 57, 2853−2859.


Acute effects of polyphenols from cranberries and grape seeds on endothelial function and performance in elite athletes: Randomized, placebo-controlled, cross-over studies. Sports. 2013.
Acute effects of polyphenols from cranberries and grape seeds on endothelial function and performance in elite athletes: Randomized, placebo-controlled, cross-over studies

Kim Labonté 1, Charles Couillard 1, Annie Motard-Bélanger 1, Marie-Eve Paradis 1, Patrick Couture 1 and Benoît Lamarche 1,*

1 The Institute on Nutrition and Functional Foods (INAF), Laval University, Québec, G1V 0A6, Canada; E-Mails: kim.labonte@videotron.ca (K.L.); Charles.couillard@fsaa.ulaval.ca (C.C.); Annie.MotardBelanger@dfc-plc.ca (A. M.-B.); marie-eve.paradis@fsaa.ulaval.ca (M.-E.P.); patrick.couture@crchul.ulaval.ca (P.C.)

* Author to whom correspondence should be addressed; E-Mail: benoit.lamarche@inaf.ulaval.ca (B.L.); Tel.: +1-418-656-2131 x.4355; Fax: +1-418-656-5877.

Received: ; in revised form: / Accepted: /

Published:

Abstract: We examined how intake of polyphenols modifies brachial artery flow-mediated dilation (FMD) at rest and cycling performance in elite athletes. In a first randomized cross-over study, FMD was measured over a three hour period on two occasions in 8 elite male and female speed skaters after acute consumption of either polyphenols from cranberries and grape seeds (600 mg) or a polyphenol-free placebo drink. In a second study, 12 male and female athletes completed a 3-km time trial (TT) on an ergocycle on two occasions in random order either after consumption of 800 mg of polyphenols or a placebo. Acute consumption of the polyphenol-rich drink led to a significant increase in FMD compared to placebo (p=0.02), with a peak at 60 minutes. Acute intake of the polyphenol extract had no impact on the 3 km time trial completion. However, plasma lactate levels were significantly lower after the TT when subjects consumed the polyphenols vs. placebo (p<0.05). Results suggest that polyphenols from cranberries and grape seeds acutely modifies FMD at rest in elite athletes. Although this does not translate into enhanced cycling performance, it may reflect accelerated lactate clearance after a maximum effort.

Keywords: polyphenol; athlete; endothelial function; FMD.
1. Introduction

Berry fruits contain a large variety of polyphenolic compounds that have been characterized as having numerous physiological effects in humans. Intake of dietary polyphenols not only enhances whole body antioxidant capacity [1], but also modulates endothelial function [2]. Polyphenols from various sources have also been shown to have anti-inflammatory properties in non-athletes and in patients with coronary heart disease [3].

Increased oxygen consumption through physical activity and exercise training, particularly at high intensities, leads to a transient pro-oxidative and pro-inflammatory state [4]. There is also accumulating evidence suggesting that oxidative stress may hamper sports performance, induce fatigue and delay recovery [5, 6, 7]. This has led many athletes to use antioxidant supplements, despite the fact that there is currently no convincing evidence supporting the beneficial effects of antioxidant supplementation on performance and exercise recovery [8].

On the other hand, it is not clear how dietary intake of polyphenols affects endothelial function in athletes, as most studies that have investigated this topic have been conducted in non-athletes as well as patients with chronic disease. Furthermore, the potential of polyphenols to improve blood flow must not be overlooked as restricted blood flow is considered a key limiting factor in muscle oxygenation during high intensity efforts [9]. For instance, it has been shown that restricted blood flow is an important determinant of performance in speed skaters [10]. However, information remains scarce regarding the impact of nutritional products on performance via their impact on vascular function,
endothelial function and blood flow [11]. It also is not clear how dietary intake of polyphenols affects endothelial function in athletes, as most studies that have investigated this topic have been conducted in non-athletes as well as patients with chronic disease.

To the best of our knowledge, no study has yet investigated the impact of acute polyphenol supplementation on vascular function and performance of elite athletes. The general objective of this study was to test the impact of a polyphenol extract from grape seed and cranberries on endothelial function and performance in elite athletes.

2. Subjects and Methods

This investigation comprised two studies each undertaken according to a randomized, double blind, placebo-controlled, cross-over design. In the first study, flow-mediated dilation (FMD) at rest was measured over a three-hour period on two occasions in elite athletes after acute consumption of a sports drink added with a proprietary blend of polyphenols from cranberries and grape seeds vs. a carbohydrate-matched polyphenol-free placebo drink. In the second study, the performance of athletes on a 3-km time trial (TT) was investigated on two occasions, i.e. after consumption of the polyphenol-rich drink vs. after consumption of a placebo.

2.1. Population

Elite athletes living in the Quebec City Metropolitan area were recruited to participate in this study. To be included in the study, subjects had to train at a high level in their sport (more than 20 hours a week), had to be free of cardiovascular diseases, diabetes or endocrine disorders, hypercholesterolemia, iron-deficiency or megaloblastic anemia (according to their physician), excessive alcohol consumption (more than two drinks by day for men, one for women) or allergies to cranberry. Data on VO2max were not collected nor available. Subjects gave their written consent to participate in this study, which was approved by the Laval University Ethics Committee.

2.2. Nutritional habits

Before both studies, subjects completed a validated food frequency questionnaire [12] with a registered dietician to assess usual food intake and to provide nutritional guidance specific to the purposes of the study. Subjects were asked to avoid cranberry juice and derived products during the entire experimental periods while also limiting their intake of polyphenols-rich foods (e.g. berries, onions, broccoli, apples, red grapes and fruit juices). The use of polyphenol supplements was strictly forbidden during both studies. Coffee and tea were not permitted on tests days. Participants also had to refrain from drinking alcoholic beverage 72 hours prior to each test.

2.3. Study designs

2.3.1. Study 1: Polyphenols and FMD in elite athletes

Acute changes in FMD were measured after administration of the following treatments in random order 1) 600 mg of a proprietary blend of polyphenols from cranberries (Vaccinium macrocarpon) and
grape seeds (*Vitis vinifera*) commercialized as CranLoad™, mixed in 500 mL of water and 2) after polyphenol-free placebo drink (500 ml). Both beverages contained equal quantities of carbohydrates (6%) from identical sources. FMD was assessed over a 3-hour period at predetermined time points (0, 30, 60, 90, 120, 150, 180 minutes).

Flow mediated vasodilation (FMD) was assessed by ultrasonography, as previously described [13]. Briefly, the brachial artery was scanned in the longitudinal plane, above the antecubital fossa with a high-resolution, linear array ultrasonic transducer of 7.5–10.5MHz (Hewlett-Packard, Sonos 5500, Andover, Massachusetts, USA). Reactive hyperaemia was induced by inflating a forearm occlusive cuff to 220 mm Hg for 3 min. Brachial artery diameter at each time point was measured 60 s and 90 s after cuff deflation. Because both measurements (60 and 90s) yielded very similar results, the mean of the two assessments was used in the present analysis. All scans were performed by the same examiner throughout the study. Images were digitally acquired and analyzed offline by two independent observers, who were blinded to the subject’s treatment assignment. Data from each person were comparable and therefore the mean of the two independent assessments was used for the analysis. FMD was calculated as the percentage increase in hyperaemia-induced brachial artery diameter from values measured at rest. Both tests (placebo and polyphenol-rich drink) were undertaken one week apart in each athlete but on the same day of their weekly training program. FMD tests were performed in the morning in a fasting state. One subject had to be excluded from the analysis because of the quality of the images collected, which did not allow the measurement of arterial diameter with reliability. Analysis of FMD in response to the test drinks was therefore performed on a total of 8 subjects.

Plasma measurements were taken over the 3-hour FMD test at predetermined time points (0, 15, 30, 45, 60, 90, 120, 150, 180 minutes) for the assessment of plasma glucose, insulin and C-peptide, using a catheter inserted in the opposite arm. Blood pressure and heart rate were also monitored during the tests.

### 2.3.2. Study 2: Polyphenols and Performance

Participants completed 3-km cycling time trials (TT) in order to investigate the impact of polyphenol intake on acute peak performance. For that purpose, participants came to the Physical Activity Sciences Laboratory of Laval University on three separate occasions, each time after lunch. The first test was undertaken in order for athletes to get familiar with the experimental procedures and the nature of the test. The 3-km TT was repeated twice thereafter by all participants in random order, i.e. once after the consumption of 800 mg of the polyphenol extract taken with 60 mL of water, and once after the consumption of 60 ml of a flavor-matched placebo drink that contained no polyphenols. The dose of polyphenols in the performance test was increased (800 vs. 600 mg in Study #1) to maximize the change in FMD even further and thereby increasing the probability to obtain a significant result on performance. No carbohydrate was added to these experimental drinks. Because the 3-km TT was performed after lunch, nutritional directions were given to subjects to ensure that they consumed the same breakfast, lunch and snack before each test was comparable. Each test was separated by one week and participants as well as all the laboratory staff were blinded from treatment assignment.
The 3-km TTs were performed on a Velotron cyclePro (RacerMate Inc., Seattle, WA, USA). The polyphenol-rich drink and the placebo were taken 60 minutes prior to the 3-km TT. Subjects had a 30 minutes period for warm up, during which they were only allowed to drink water. Athletes were allowed to select their own pace and wheel resistance, which they could change freely during the test and also had the possibility to pedal in a standing rather than sitting position. The 3-km test was run on a simulated flat course. Time to completion was the primary outcome. Other measures included generated wattage, heart rate, plasma lactate levels and perceived exertion. Plasma lactate concentrations were measured after the warm up as well as right at the end of the 3-km TT and 2.5 and 5 minutes post TT with a finger stick device and a lactate meter (Lactate Pro LT-1710, Fact-Canada, Quesnel, BC, Canada). Perceived exertion was assessed both for globally perceived effort as well as specifically for legs and breathing using the 20-point Borg scale. During each test, coaches and training partners were allowed to support the participants. Heart rate was monitored throughout the test with an electrode strap belt. Prevalence of side-effect was assessed by a questionnaire completed on the day that followed each 3-km TT test.

2.4. Statistical analyses

Data are expressed as mean ± standard deviation unless specified otherwise. Between treatments comparisons in both studies were tested using mixed models for repeated measurements (SAS software version 9.1). A P value less than 0.05 was considered statistically significant.

3. Results

Eighteen of the 20 athletes whose data are included in this study were members of the Canadian Development or National Speed Skating Team. Characteristics of the participants are presented in Table 1.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Study 1</th>
<th>Study 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Females (N)</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>23.4 ± 7.7</td>
<td>22.0 ± 2.4</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>70.8 ± 6.7</td>
<td>75.7 ± 10.4</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.75 ± 0.08</td>
<td>1.79 ± 0.07</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.2 ± 0.8</td>
<td>23.6 ± 1.9</td>
</tr>
<tr>
<td>Energy (kcal)</td>
<td>3931 ± 1347</td>
<td>3139 ± 735</td>
</tr>
<tr>
<td>% from carbohydrates</td>
<td>57 ± 7</td>
<td>55 ± 6</td>
</tr>
<tr>
<td>% from protein</td>
<td>16 ± 2</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>% from fat</td>
<td>26 ± 5</td>
<td>28 ± 6</td>
</tr>
</tbody>
</table>

Values are presented as mean ± standard deviation. BMI: body mass index.
Study 1- Polyphenols and FMD

Intake of the polyphenol-rich drink significantly increased FMD at times 30, 60, 90 and 120 minutes following hyperaemia compared with placebo (P<0.05) (Figure 1). FMD peaked in average at 60 minutes. Mean FMD returned to baseline values 3 hours after ingestion of the polyphenol rich drink. As a result, the area under the curve of FMD over time was greater after the polyphenol-rich drink than after the placebo (P=0.02). As shown in Figure 2, there was no difference in plasma glucose, insulin and C-peptide responses to the polyphenol-rich and placebo drinks.

Figure 1. Flow-mediated dilation (FMD) variation from baseline after intake of the polyphenol-rich drink (grey symbols) and placebo (black symbols). *P<0.05 versus baseline and placebo at specified time points. The insert presents the area under the curve (AUC) of the change in FMD over time above baseline (time 0) values. The Y axis is in %*min.
Figure 2. Glycemia, insulinemia and C-Peptide concentration at baseline and after the ingestion of the polyphenol-rich drink or placebo. The inserts present the area under the curve (AUC) of the change over time above baseline (time 0) values. The Y axis for the inserts is in mmol/l*min. There was no statistical difference between treatments.
Study 2- Polyphenols and performance

Time to completion of the 3-km TT was virtually identical between the 2 treatments (267.5 ± 24.5 sec vs. 265.7 ± 21.4 sec with the polyphenol-rich and placebo treatments respectively) and the difference between the two (1.75 sec, 0.66%) was not significant (p=0.27). There was also no difference in average wattage and perceived exertion after the TT between the polyphenol-rich and placebo drinks. However, consumption of polyphenols 60 minutes before the TT was associated with lower plasma lactate concentrations both before the onset of the test (-30.3%) as well as immediately following the test (-7.9%) and 2.5 minutes post recovery (-4.8%, Figure 3, p<0.05). Heart rates (HR) at the end of the 3-km TT were comparable after both conditions (polyphenol-rich drink: 188±6 bpm, placebo: 190±6 bpm, p=0.22). However, HR was significantly lower 2 min post exercise (140±10 vs. 144±9 bpm, p=0.04, N=11) and 5 minutes post exercise (120±7 vs. 122±8 bpm, p=0.05, N=10) after the polyphenol-rich drink vs. placebo. No side effect was reported by the athletes in response to either treatment.

Figure 3. Blood lactate levels before and immediately after the 3-km time trial (TT) test and 2.5 min and 5 min post effort.
4. Discussion

The present study suggests that consumption of a polyphenol-rich drink significantly increases FMD in elite athletes. Although this change in vascular function is not associated with enhanced cycling performance, lower blood lactate and heart rate post TT associated with polyphenol consumption may reflect accelerated recovery.

Study 1 - Polyphenols and FMD

The significant increase in acute FMD following consumption of the polyphenol-rich drink is consistent with other studies conducted in non-athlete populations [14, 15, 16, 17]. For instance, FMD was increased after consumption of 477 mg of polyphenols from non-alcoholised red wine in healthy subjects [16]. Coronary artery disease (CAD) patients showed a 2.1% improvement in FMD measured at 60 minutes after intake of non-alcoholised wine [18]. In another study involving CAD patients [19], FMD after consumption of 600 mg of polyphenols from red wine peaked at 60 minutes, which is similar to our results in elite athletes. However, maximal improvement in FMD (1.9% increase from baseline) with polyphenol intake was slightly smaller than the change seen in our population of athletes (2.8% increase from baseline). This difference in magnitude may be attributable to age differences in subjects between the two studies (mean age of 61 vs. 23 years), since endothelial function tends to deteriorate with aging [20]. Fitness level of subjects is another key determinant of endothelial function and FMD [20]. Nevertheless, results from the present study suggest that even in highly trained athletes, consumption of 600 mg of polyphenols from cranberries and grape seeds modifies FMD acutely.

Mechanisms underlying the acute FMD response to polyphenol intake have been proposed. There is in vitro evidence for changes in the phosphorylation pattern of endothelial nitric oxide synthase (eNOS) in response to tea polyphenols [21]. Consistent with these data, treatment of cultured bovine aortic endothelial cells (BAEC) with hesperetin, a citrus flavonoid, has been shown to acutely stimulate phosphorylation of eNOS to produce nitric oxide, a key signalling molecule involved in vasodilation. TNF-α mediated adhesion of monocytes to BAEC has also been show to be attenuated by treatment with hesperetin [22]. This suggests that pre-existing signalling pathways rather than changes in gene expression and protein synthesis are most likely responsible for the rapid and acute FMD response to a single dose of polyphenol [23].

Chronic intake of flavonols and antioxidants has been suggested to attenuate insulin resistance in various populations [24, 25, 26] but we are not aware of studies that have investigated this issue in the context of an acute consumption in highly trained individuals. Our results have shown that a single 600 mg dose of a polyphenol mix from cranberries and grape seeds does not alter the glycemic and insuliniemic response to carbohydrate intake in this population, even when FMD is significantly altered. It will be interesting to investigate if chronic intake of polyphenols has long-term effects on these parameters.

Study 2 - Polyphenols and performance

It was of interest as part of our second objective to investigate if modifying FMD is associated with enhanced performance. We found that acute intake of polyphenols in elite athletes had no impact on
performance in a 3-km bike TT as well as on perceived exertion. Participants received strict recommendations regarding intake of polyphenol-rich foods and supplements during the study. Based on the randomized cross-over nature of the study and its overall short duration (1 week separated the 2 test days), usual dietary intake of polyphenols from polyphenol-rich foods is unlikely to be a confounding factor in our study.

The absence of an effect on performance can be attributed to several factors. First, it is possible that the dose of polyphenols used (800 mg) may have led to changes in vascular function that are too small in magnitude to influence performance on a maximal test. Second, it has been shown that vascular function deteriorates in soccer players during the playing season, and returns to normal levels after a 4 weeks recovery period [27]. Study 2 was undertaken when athletes had just started their training regimen after a one-month rest. Since vascular function may degrade as the training amplifies during the season, testing the impact of polyphenol intake on performance during a more intense training period is of great relevance. Finally, the duration of the TT was approximately 4 minutes. We cannot exclude the possibility that a single dose of polyphenols through its vasodilating effect may affect performance in the context of longer duration events. Indeed, acute supplementation with polyphenols from *Ecklonia cava* in 23 college student volunteers significantly increased time to exhaustion on a bicycle compared with placebo [28]. This deserves further investigation in elite athletes.

On the other hand, our results also suggest that for a similar degree of effort, time on the ergocycle and perceived exertion, a single dose of polyphenols pre-exercise results in significantly lower blood lactate concentration and heart rate post effort. Results were relatively similar in men and women (not shown). The increase in time to exhaustion after intake of polyphenols from *Ecklonia cava* was seen along with a non-significant trend towards a decrease in plasma lactate levels in college students [28]. In a chronic 4-week supplementation study with 800 mg of a coffeeberry formulation, recovery blood lactate concentrations were significantly reduced compared with placebo, with no significant effect of the supplementation, however, on anaerobic performance [29]. The apparent reduction in post exercise blood lactate levels and in heart rate when polyphenols are consumed prior to a maximal effort may reflect physiological mechanisms associated with favourable recovery processes.

8. Conclusion

Data from the present study show that acute polyphenol supplementation improves endothelial function but has no impact on performance on a 3-km TT. On the other hand, physiological responses to short-term maximal exercise after a single dose of polyphenols suggest favourably altered recovery capacity. It is tempting to hypothesize that altering FMD through intake of polyphenols may facilitate muscle irrigation and thereby recovery as well, by modifying the clearance of metabolites produced during submaximal and maximal efforts. Additional studies are needed to see how chronic vs. acute intake of polyphenols in athletes affects performance, but also recovery using specific and recognized endpoints. Finally, polyphenols have well characterized antioxidant properties [1]. The impact of antioxidant supplementation on sports performance is highly debated, but it should be stressed that most studies on this topic have been conducted using relatively weaker antioxidant supplements such as vitamins [30, 31, 32]. It will be interesting to balance the FMD-related effects of polyphenols with their antioxidant-related effects on recovery and performance.
Acknowledgments

These studies were developed and supported by Speed Skating Canada through the Own the Podium Program towards the 2010 Olympic Games in Vancouver and by Atrium Innovation. We are grateful to the nurses and the laboratory staff of the Nutrition and Functional Foods Institute and of Laboratory of Science of Physical Activity for their technical assistance and the expert care provided to the participants. We also express our gratitude to the participants, without whom the study would not have been possible.

Author’s contribution

BL and CC has designed and obtained funding for this study. PC was responsible for the screening and medical supervision of the study participants. AMB coordinated Study #1 and KL coordinated Study #2. MEP was involved in manuscript preparation and revision. BL and KL performed statistical analyses, analyzed the data and wrote the manuscript, which was reviewed critically by all authors. PC are Research Scholars from the Fonds de la recherche en santé du Québec (FRSQ). Laval University, the employer of BL and CC, has licensed the product tested in these studies (CranLoad™) to Atrium Innovations, for commercial purposes.
References and Notes


© 2013 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/3.0/).
Effects of polygonum cuspidatum containing resveratrol on inflammation in male professional basketball players.
ABSTRACT

Background: Exercise can lead to acute oxidative stress, which can result in oxidative damage and induce inflammation. Resveratrol may reduce the levels of inflammatory cytokines. Thus, we investigated the effects of this compound on the plasma levels of tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) in male professional basketball players.

Methods: Twenty healthy male professional basketball players were randomized into two groups (10 each). For 6 weeks, they received daily either 200 mg of polygonum cuspidatum extract (PCE) standardized to contain 20% trans-resveratrol equivalent to 40 mg trans-resveratrol or placebo. Indices of inflammation were measured before and after 6 weeks of supplementation.

Results: There was a significant reduction in plasma levels of TNF-α and IL-6 after 6 weeks of supplementation; while no change was observed in these markers in the control group.

Conclusions: Present study shows that 6 weeks of PCE containing resveratrol supplementation reduces the inflammation in male professional basketball players.

Keywords: Cytokines, interleukin-6, inflammation, polygonum cuspidatum, resveratrol, tumor necrosis factor-α

INTRODUCTION

It is now recognized that both acute aerobic and anaerobic exercise can cause production of free radicals that lead to acute oxidative stress, which can result in oxidative damage and induce inflammation.[1]

Recent studies have demonstrated that there is a link between plasma concentration of inflammatory mediators and pathogenesis of insulin resistance, hypertension, obesity, and complications of diabetes such as retinopathy.[2,3] Furthermore, there are some evidences about the association of low-grade inflammation and cardiovascular risk.[4]

Resveratrol is a natural anti-oxidant polyphenol that is present in red wines, grapes, and roots of polygonum cuspidatum that has received noticeable attention in recent years.[5,6] This strong
polyphenolic compound has shown several biological functions such as anti-inflammatory and anti-oxidant. It has been revealed to exert some health-enhancing properties like protection against cardiovascular disease and inhibition of cancer. So far, most studies about resveratrol have focused on animal models; however, there are some works shown the beneficial effects of this compound on human, however, its strong anti-inflammatory properties have not been investigated in professional athletes. Thus, we have hypothesized that the extracts of polygonum cuspidatum containing resveratrol can reduce inflammation levels in male professional basketball players.

The aim of this study was to investigate the effects of polygonum cuspidatum extract (PCE) containing resveratrol on inflammation in male professional basketball players.

METHODS

Twenty healthy professional basketball players (aged 17-35 years) were randomized into two groups (10 each). For 6 weeks, they received either 200 mg of PCE standardized to contain 20% trans-resveratrol equivalent to 40 mg trans-resveratrol (Pure Encapsulations Inc., Sudbury, MA) or placebo daily. The subjects were instructed not to take any anti-oxidant supplements, and anti-inflammatory drugs during, and 2 weeks before the study. Exclusion criteria included the incidence of any diseases especially those that involve immune system.

Venous blood samples were collected after and 2 h of intensive endurance exercise between 6 and 7 pm at the baseline and after 6 weeks of treatment with resveratrol. Ethical approval from the Medical Ethics Committee of Tehran University of Medical Sciences was obtained and participants signed informed consent.

The serum levels of tumor necrosis factor-α (TNF-α), and interleukin 6 (IL-6) were measured by enzyme immunometric assay kits using the kits of assay designs (Ann Arbor, MI). Nutritionist 4 (First Data Bank, San Bruno, CA, USA) was used to perform nutrient calculations for 3d dietary records that obtained before and after the intervention. The statistical tests were conducted using SPSS (version 16; SPSS, Inc., Chicago, IL, USA). Data were represented as means and standard deviations. P <0.05 was considered as statistically significant.

RESULTS

Twenty-four athletes were recruited but 20 of them completed the intervention for 6 weeks. Incidence of diseases and personal reasons were the main reasons of their withdrawal of the study. Baseline characteristics and some nutrients intake of participants are shown in Table 1. Mean (SD) plasma levels of TNF-α and IL-6 are seen in Table 2.

As Table 1 shows, there were no significant differences between the groups with regard to weight, body mass index and dietary intake. IL-6 and TNF-α decreased significantly in resveratrol group and there were significant differences between the two groups after intervention [Table 2].

DISCUSSION

The present study, investigated the effect of PCE containing resveratrol on plasma level of TNF-α, and IL-6 in male professional basketball players. In this randomized double-blind placebo-controlled clinical trial study, intake of PCE containing resveratrol for 6 weeks was demonstrated to reduce the plasma concentration of TNF-α and IL-6 significantly (P < 0.05). These findings confirmed the results of previous studies of Bujanda et al.,[9] Ghanim et al.,[10,11] in rats and healthy humans. In 2008, Bujanda et al. demonstrated that production of TNF-α decreased in rats treated with resveratrol.[19] Furthermore, this author proposed that anti-TNF-α effect of resveratrol could be related to decreasing liver damage in a model of liver steatosis. Ghanim et al. in 2010 found that intake of PCE containing resveratrol suppressed plasma concentration of TNF-α, IL-6, and C-reactive protein after 6 weeks in healthy humans while they did not observe any changes in these indices in the control group.[10] On the other hand, Ghanim et al. in 2011 have shown the anti-inflammatory effects of resveratrol and polyphenol preparation supplement in healthy humans.[11]

It is now recognized that both acute aerobic and anaerobic exercise can cause production of free radicals that lead to acute oxidative stress, which can result in oxidative damage and induces inflammation.[1] In addition, strenuous exercise can lead to sequential release of TNF-α and IL-6 in the blood that is comparable to that observed in relation to bacterial diseases.[12]
One of the suggestive mechanisms for this effect includes down-regulation of inflammatory response via inhibition of production and release of pro-inflammatory markers by its suppressive effect on nuclear factor-κB or the activator protein.\textsuperscript{[13–15]}

To our knowledge, this is the first study to investigate the effects of this supplement in professional basketball players; however, there were some limitations. The major limitation is that it has been conducted in a small number of professional athletes because of the limitation in accessing to them. Another one is the short length of the intervention. On the other hand, the blood level of resveratrol was not measured in this study.

Table 1: Baseline characteristics and some nutrients intake throughout the study\textsuperscript{a}

<table>
<thead>
<tr>
<th>Variables</th>
<th>Group</th>
<th>Baseline</th>
<th>After intervention</th>
<th>$P$ value$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>Resveratrol</td>
<td>95.73±15.83</td>
<td>95.97±15.17</td>
<td>0.397</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>90.6±8.94</td>
<td>90.53±9</td>
<td>0.553</td>
</tr>
<tr>
<td>$P$ value$^c$</td>
<td></td>
<td>0.38</td>
<td>0.447</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>Resveratrol</td>
<td>25.25±3.22</td>
<td>25.33±3.06</td>
<td>0.387</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>26.88±5.51</td>
<td>26.86±5.46</td>
<td>0.38</td>
</tr>
<tr>
<td>$P$ value</td>
<td></td>
<td>0.43</td>
<td>0.315</td>
<td></td>
</tr>
<tr>
<td>Energy (kcal)</td>
<td>Resveratrol</td>
<td>2492.6±354.28</td>
<td>2558.2±362.68</td>
<td>0.551</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>2564.5±340.71</td>
<td>2572.7±398.43</td>
<td>0.775</td>
</tr>
<tr>
<td>$P$ value</td>
<td></td>
<td>0.64</td>
<td>0.941</td>
<td></td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>Resveratrol</td>
<td>308.06±88.5</td>
<td>321.48±86.96</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>312.07±57.9</td>
<td>309.54±65.87</td>
<td>0.752</td>
</tr>
<tr>
<td>$P$ value</td>
<td></td>
<td>0.9</td>
<td>0.701</td>
<td></td>
</tr>
<tr>
<td>Protein (g)</td>
<td>Resveratrol</td>
<td>93.35±24.57</td>
<td>85.24±17.14</td>
<td>0.317</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>90.68±18.24</td>
<td>89.96±23.38</td>
<td>0.864</td>
</tr>
<tr>
<td>$P$ value</td>
<td></td>
<td>0.78</td>
<td>0.976</td>
<td></td>
</tr>
<tr>
<td>Fat (g)</td>
<td>Resveratrol</td>
<td>101.06±17.12</td>
<td>111.66±17.66</td>
<td>0.537</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>105.01±16.37</td>
<td>113.07±16.3</td>
<td>0.48</td>
</tr>
<tr>
<td>$P$ value</td>
<td></td>
<td>0.19</td>
<td>0.279</td>
<td></td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>Resveratrol</td>
<td>76.56±55.48</td>
<td>81.3±62.07</td>
<td>0.319</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>67.56±47.5</td>
<td>81.1±39.99</td>
<td>0.166</td>
</tr>
<tr>
<td>$P$ value</td>
<td></td>
<td>0.7</td>
<td>0.609</td>
<td></td>
</tr>
<tr>
<td>Vitamin E (mg)</td>
<td>Resveratrol</td>
<td>28±3.75</td>
<td>28.4±4.22</td>
<td>0.525</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>25.78±3.18</td>
<td>26.2±3.3</td>
<td>0.099</td>
</tr>
<tr>
<td>$P$ value</td>
<td></td>
<td>0.17</td>
<td>0.189</td>
<td></td>
</tr>
<tr>
<td>β-carotene (μg)</td>
<td>Resveratrol</td>
<td>408.91±509.51</td>
<td>414.1±508.98</td>
<td>0.402</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>325.01±522.07</td>
<td>201.85±306.03</td>
<td>0.347</td>
</tr>
<tr>
<td>$P$ value</td>
<td></td>
<td>0.8</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>Zinc (mg)</td>
<td>Resveratrol</td>
<td>10.25±2.12</td>
<td>11.02±1.69</td>
<td>0.402</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>10.23±1.68</td>
<td>10.98±2.42</td>
<td>0.083</td>
</tr>
<tr>
<td>$P$ value</td>
<td></td>
<td>0.97</td>
<td>0.708</td>
<td></td>
</tr>
<tr>
<td>Selenium (mg)</td>
<td>Resveratrol</td>
<td>0.05±0.02</td>
<td>0.05±0.01</td>
<td>0.469</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>0.04±0.02</td>
<td>0.05±0.02</td>
<td>0.313</td>
</tr>
<tr>
<td>$P$ value</td>
<td></td>
<td>0.28</td>
<td>0.604</td>
<td></td>
</tr>
<tr>
<td>Fiber (mg)</td>
<td>Resveratrol</td>
<td>14.8±4</td>
<td>16.04±4.86</td>
<td>0.328</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>15.03±5.62</td>
<td>14.81±5.08</td>
<td>0.61</td>
</tr>
<tr>
<td>$P$ value</td>
<td></td>
<td>0.93</td>
<td>0.448</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}Data are presented as mean±standard deviation. BMI=Body mass index, \textsuperscript{b}To test for statistical difference between the two study groups independent-samples $T$ test was used. \textsuperscript{c}To test for statistical difference between two intervals within a group paired-samples $T$ test was used.

www.mui.ac.ir
Table 2: Tumor necrosis factor-α and interleukin-6 levels of participants during the study

<table>
<thead>
<tr>
<th>Variables</th>
<th>Group</th>
<th>Baseline (pg/mL)</th>
<th>After intervention (pg/mL)</th>
<th>P valuec</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>Resveratrol</td>
<td>9.73±0.25</td>
<td>9.31±0.2</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>9.78±0.15</td>
<td>9.83±0.25</td>
<td>0.322</td>
</tr>
<tr>
<td>IL-6</td>
<td>Resveratrol</td>
<td>75±8.3</td>
<td>70.8±7.27</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>79.5±8.21</td>
<td>77.7±7.79</td>
<td>0.179</td>
</tr>
</tbody>
</table>

TNF-α=Tumor necrosis factor-α, IL-6=Interleukin-6.
aData are presented as mean±standard deviation. bTo test for statistical difference between two intervals within a group paired-samples T test was used. cTo test for statistical difference between the two study groups independent-samples T test was used.

CONCLUSIONS

In conclusion, this study indicates that PCE containing resveratrol has suppressive effects on some of immune system factors, including TNF-α and IL-6 plasma levels.

ACKNOWLEDGMENTS

The present study was supported by Vice-Chancellor for Research; Tehran University of Medical sciences. We thank Mr. Hatami and Mr. Salehi for their assistance in the basketball teams and Miss Chamari for nutrients intake analysis. The authors declared that they have no conflict of interest.

REFERENCES


Source of Support: This study was conducted as a thesis funded by Tehran University of Medical Sciences, Tehran, Iran.
Conflict of Interest: None declared.
Anti-inflammatory and neuroactive properties of selected fruit extracts.
Anti-Inflammatory and Neuroactive Properties of Selected Fruit Extracts

Kelly C. Heim, Paul Angers, Sebastien Léonhart, and Barry W. Ritz

1Pure Encapsulations, Inc., Sudbury, Massachusetts, USA.
2Center of Horticulture Research, Laval University, Quebec City, Quebec, Canada.
3NutraCanada, Champlain, Quebec, Canada.
4Atrium Innovations, Inc., Chadds Ford, Pennsylvania, USA.

ABSTRACT Epidemiological evidence supports inverse associations between fruit and vegetable intake and incidence of cardiovascular disease and neurodegeneration. Dietary botanicals with salient health benefits include berries and leafy vegetables. Molecular pharmacology research has ascribed these benefits primarily to phenolic constituents and antioxidant activity. The current investigation sought to elucidate pharmacologic activity of two novel preparations of berry and spinach extracts in vitro. Blueberry and cranberry exhibited the greatest antioxidant activity. In a dose-dependent manner, a proprietary mixture of cranberry and blueberry extracts inhibited inhibitor of κB kinase β, a central node in inflammatory signal transduction. A proprietary mixture of blueberry, strawberry, and spinach extracts inhibited prolyl endopeptidase, a regulator of central neuropeptide stability and an emerging therapeutic target in neurology and psychiatry. These results indicate specific molecular targets of blended dietary plants with potential relevance to inflammation and neurological health.

KEYWORDS: anti-inflammatory • antioxidant • cranberry • polyphenol • strawberry

CLINICAL EVIDENCE SUGGESTS that cranberry (Vaccinium macrocarpon), blueberry (Vaccinium angustifolium), strawberry (Fragaria vesca), and spinach (Spinacia oleracea) confer protection against cardiovascular and neurodegenerative diseases.1–8 Mechanistic investigations have indicated that these plant foods attenuate oxidative stress, modify inflammation, and alter neurotransmission, effects that are commonly ascribed to phenolic constituents.4,5,7,9 Nuclear factor κB (NFκB) is a major inflammatory mediator associated with neurodegeneration and atherogenesis and is amenable to inhibition by many phytochemicals.10,11 NFκB is activated by phosphorylation by inhibitor of κB kinase (IKKβ), a kinase that directs the degradation of inhibitor of κB, an anchor that basally sequesters NFκB in the cytosol. Once liberated, NFκB translocates to the nucleus to orchestrate inflammatory gene expression.12 Botanical extracts with anti-inflammatory activity often exhibit neuroprotective effects through mechanisms both related to and distinct from NFκB.13 Prolyl endopeptidase (PEP) is a cytosolic peptidase that degrades central neuropeptides, including oxytocin, bradykinin, and substance P. In clinical studies, PEP inhibitors improve neurocognitive performance.14–16 To date, many NFκB studies and nearly all PEP studies have examined phytochemical isolates that directly bind and inhibit IKKβ and PEP.17–19 However, fruits and vegetables present arrays of phytochemicals, and functional interactions afforded by chemodiversity may be relevant to their health benefits.20,21 For example, a whole cranberry extract exhibited greater inhibition of cancer cell proliferation than purified polyphenolic fractions.20 The current study examines extracts containing naturally low concentrations of phenolics in mixtures more representative of dietary preparations.18,19,22 This short communication reports NFκB and PEP inhibition in vitro by two mixtures of extracts of widely consumed plant foods. Aqueous extracts of cranberry (V. macrocarpon) and wild blueberry (V. angustifolium) and ethanol extracts of Orleans strawberry (F. vesca var. Orléans) and spinach (S. oleracea) were obtained from NutraCanada (Champlain, QC, Canada). Various potential combinations of these extracts were assessed, and the final compositions of the two investigated blends are indicated in Table 1. Final extract combinations were cranberry and blueberry (CB) (supplied as PhytoCardio; Pure Encapsulations, Sudbury, MA, USA) and Orléans strawberry, blueberry, and spinach (SBS) (supplied as PhytoMemory; Pure Encapsulations).

Individual extracts of cranberry, blueberry, strawberry, and spinach were subjected to oxygen radical absorbance capacity (ORAC) assays at Laval University, Sillery, QC, Canada, according to the method of Cao and Prior.23 Table 1 gives ORAC values of the extracts.

The CB extract combination was subjected to an IKKβ inhibition experiment, conducted with an assay kit from...
Table 1. Fruit and Vegetable Blends Studied

<table>
<thead>
<tr>
<th>Blend, extract constituent</th>
<th>Phenolic content</th>
<th>ORAC (μmol of TE/g)</th>
<th>Bioassay</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cranberry</td>
<td>2% proanthocyanidins</td>
<td>350</td>
<td>NFκB inhibition</td>
</tr>
<tr>
<td>Blueberry</td>
<td>4% total phenolics</td>
<td>350</td>
<td></td>
</tr>
<tr>
<td>SBS*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strawberry</td>
<td>2% total phenolics</td>
<td>200</td>
<td>PEP inhibition</td>
</tr>
<tr>
<td>Blueberry</td>
<td>4% total phenolics</td>
<td>350</td>
<td></td>
</tr>
<tr>
<td>Spinach</td>
<td>1% total phenolics</td>
<td>300</td>
<td></td>
</tr>
</tbody>
</table>

*Cranberry/blueberry, supplied as PhytoCardio (Pure Encapsulations).
*aCranberry/blueberry/spinach, supplied as PhytoMemory (Pure Encapsulations).

NFκB, nuclear factor κB; ORAC, oxygen radical absorbance capacity; PEP, prolyl endopeptidase; TE, Trolox equivalents.

ChromaDex (Irvine, CA, USA). The HTRF® KinEASE™ kit (catalog number 62ST3PEB; Cisbio, Bedford, MA, USA) is a homogeneous time-resolved fluorescence assay based on the proximity of a europium cryptate donor label and an XL665 acceptor label. For each sample, 5 mg was dissolved in 150 μL of methanol and brought to 5 mL with water, from which dilutions were prepared. A total enzymatic reaction mixture of 10 μL contained 2 μL of kinase buffer, 2 μL of test samples or reference inhibitor at various concentrations in vehicle buffer, 2 μL of active IKKβ, 2 μL of substrate, and 2 μL of ATP. Negative and positive controls contained 10 μL of kinase buffer. Mixtures were incubated for 30 min at 37°C. We added 5 μL of XL665 and 5 μL of cryptate to the reaction, and incubation proceeded at 25°C for 1 h. The plate was read using a PHERAstar (BMG Labtech Instruments, Ortenberg, Germany) with an integration delay of 50 μs and an integration time of 400 μs. Results were calculated as follows: % inhibition = \((I/\text{Dose}) - f\%_{\text{control}}) / (f\%_{\text{control}}) \times 100\), where Δf is the ratio of absorbance at 665 nm/620 nm. The values for the concentration providing 50% inhibition (IC50) were calculated using log-probit analysis. Each of two samples and standards was tested in triplicate. Statistical significance was determined using the unpaired two-tailed t test.

SBS was subjected to a PEP inhibition assay (ChromaDex) according to the method of Kobayashi et al. PEP was measured as cleavage of the fluorogenic peptide substrate Z-Gly-Pro-AMC at the peptide bond on the carboxyl side of the proline residue. Fluorescence intensity was measured at excitation and emission wavelengths of 360 nm and 460 nm, respectively, at 30°C using a PHERAstar microplate reader. Percentage inhibition (%I) of samples were calculated from the measured relative fluorescence units (RFU) using the following equation: %I = \((\text{RFU}_{\text{control}} - \text{RFU}_{\text{sample}}) / \text{RFU}_{\text{control}}\) × 100. IC50 values were calculated from the mean percentage inhibition values using the Finney software. Each of two samples and standards was tested in triplicate. Statistical significance was determined using the unpaired two-tailed t test.

NFκB is amenable to inhibition by structurally diverse antioxidant phytochemicals through direct IKKβ inhibition and redox-dependent blockade of DNA binding. In the current study, CB exhibited a dose-dependent inhibition of IKKβ with IC50 values of 5.16 and 4.78 μg/mL for each of two samples (Fig. 1). These values indicate much lower affinity than typically reported for pure polyphenolic constituents of berries such as quercetin (IC50 = 1.35 μg/mL). IKKβ inhibition by quercetin occurs via hydrogen bonding and other interactions with the ATP binding site. Inhibition by piceatannol, a stilbene found in blueberries, is ascribed to modification of the redox state of Cys179 in the activation loop. Because several flavonoid and stilbene compounds exist in Vaccinium berries, it is possible that both types of interactions may be part of the inhibitory mechanism of CB. As CB contains numerous antioxidants, suggested by high ORAC values of blueberry and cranberry (Table 1), it may also modify other redox-sensitive points of NFκB signaling such as DNA binding. Since diverse signals converge on multiple nodes of the NFκB pathway, this partial IKKβ blockade does not necessarily predict attenuation of pro-inflammatory gene expression. Studies in different cell contexts are warranted to faithfully represent the complexities of NFκB signaling.

PEP inhibition has been reported for structurally disparate phytochemicals, suggesting that a chemically diverse extract

**FIG. 1.** Inhibition of inhibitor of κB kinase β (IKKβ) by CB. Kinase activity was measured using fluorescence resonance energy transfer. Data represent the average of two experiments with triplicate determinations. Error bars represent SEM. *P < .05 compared with all lesser concentrations in both samples.
may present multiple inhibitors at low concentrations. A dose-dependent inhibition of PEP by SBS was detected with IC_{50} values of 286.48 and 333.3 µg/mL for the two samples (Fig. 2). These values indicate much lower affinity than that of quercetin (IC_{50} = 12 µg/mL). If flavonoids are accountable for inhibition by SBS, a noncompetitive mode is likely. A critical consideration for future work is the capability of the active constituents of SBS to enter the central nervous system at therapeutically relevant concentrations. Contingent on this performance, PEP may constitute one of the targets of isolated phytochemicals are inhibited by mixtures of whole extracts. It is important that this is the first report of PEP inhibition by fruit and vegetable extracts and warrants further interrogation of these foods in neurocognitive models.

ACKNOWLEDGMENTS

This work was funded by the Quebec Ministry of Agriculture, Fishery and Food, NutraCanada, and Atrium Innovations, Inc.

AUTHOR DISCLOSURE STATEMENT

S.L. is employed by NutraCanada, which supplied the extracts. K.C.H. is employed by Pure Encapsulations, Inc., the manufacturer of dietary supplements containing CB and SBS. B.W.R. is affiliated with its parent company, Atrium Innovations. P.A. declares no competing financial interests.

REFERENCES


An anti-inflammatory and reactive oxygen species suppressive effects of an extract of polygonum cuspidatum containing resveratrol.

J Clin Endocrinol Metab. 2010.
An Antiinflammatory and Reactive Oxygen Species Suppressive Effects of an Extract of Polygonum Cuspidatum Containing Resveratrol

Husam Ghanim, Chang Ling Sia, Sanaa Abuaysheh, Kelly Korzeniewski, Priyanka Patnaik, Anuritha Marumganti, Ajay Chaudhuri, and Paresh Dandona

Division of Endocrinology, Diabetes, and Metabolism, State University of New York at Buffalo and Kaleida Health, Buffalo, New York 14209

Background: Resveratrol have been shown to exert an antiinflammatory and antiaging effects in vitro and in animal models.

Objective: The objective of the study was to investigate the effect of a Polygonum cuspidatum extract (PCE) containing resveratrol on oxidative and inflammatory stress in normal subjects.

Research Design and Methods: Two groups (10 each) of normal-weight healthy subjects were randomized to placebo or PCE containing 40 mg resveratrol daily for 6 wk. Fasting blood samples were obtained prior to and after treatment at 1, 3, and 6 wk. Mononuclear cells were prepared for reactive oxygen species generation, RNA isolation, nuclear extract, and total cell homogenate preparation. Indices of oxidative and inflammatory stress, suppressor of cytokine signaling-3, phosphotyrosine phosphatase-1B, jun-N-terminal kinase-1, and inhibitor of κB-kinase-β were measured by RT-PCR and Western blotting.

Results: The extract induced a significant reduction in reactive oxygen species generation, the expression of p47phox, intranuclear nuclear factor-κB binding, and the expression of jun-N-terminal kinase-1, inhibitor of κB-kinase-β, phosphotyrosine phosphatase-1B, and suppressor of cytokine signaling-3 in mononuclear cells when compared with the baseline and the placebo. PCE intake also suppressed plasma concentrations of TNF-α, IL-6, and C-reactive protein. There was no change in these indices in the control group given placebo.

Conclusions: The PCE-containing resveratrol has a comprehensive suppressive effect on oxidative and inflammatory stress. (J Clin Endocrinol Metab 95: E1–E8, 2010)

Oxidative stress and inflammation are involved in the pathogenesis of atherogenesis, micro- and macrovascular complications of diabetes, insulin resistance, and aging. Deletion of genes inducing oxidative and inflammatory stress reduces atherogenesis and restores insulin sensitivity and prolongs life in animal models (1–3). Transgenic animals, like the Drosophila with an excess of genes that reduce oxidative stress-catalase and superoxide dismutase, also have a prolonged life span (4, 5). Caloric restriction has also been shown to extend life span in small mammalian species (6, 7). Our work and that of others have demonstrated that caloric restriction leads to a marked reduction in oxidative and inflammatory stress in humans (8–10).

Recent work shows that resveratrol has been shown to exert an antiinflammatory and antioxidative stress in vitro and in animal models (11, 12). Resveratrol has also been shown to prolong life expectancy and reduce the rate of aging in the yeast and lower animals like yeast, Caenorhabditis elegans and Drosophila (13, 14). Resveratrol actions are thought to be mediated by increased expression of sirtuin (SIRT)-1 (13), a gene associated with lon-
gevity. SIRT-1 overexpression and resveratrol cause a reduction in the expression of phosphotyrosine phosphatase (PTP)-1B (15, 16). PTP-1B has been shown to be induced by inflammation and plays a major role in insulin resistance (17, 18). Thus, it is important to establish whether such a compound has properties that reduce oxidative and inflammatory stress in human.

Because there are no data demonstrating the effect of resveratrol on oxidative and inflammatory stress, in vivo, we have now hypothesized that Polygonum cuspidatum extract (PCE)-containing resveratrol reduces the level of oxidative and inflammatory stress in the human. Because several of the key mediators of insulin resistance that interfere with insulin signal transduction are also proinflammatory, we also investigated the effect of PCE intake on their expression.

Subjects and Methods

Subjects

Two groups (10 each) of normal-weight, age-matched healthy subjects (aged 36 ± 5 yr, body mass index 21.8 ± 0.5 kg/m²) were randomized to receive either 200 mg of PCE standardized to contain 20% trans-resveratrol (equivalent to 40 mg/d trans-resveratrol; Pure Encapsulations Inc., Sudbury, MA) or placebo daily for 6 wk. The total phenolics and flavonoid content of PCE is approximately 60% (wt/wt) and 6% (wt/wt), respectively (19). The subjects were not on any antiinflammatory drugs. They presented at the Clinical Research Center of the Diabetes Endocrinology Center of Western New York after an overnight fast at 0800–0900 h. Fasting blood samples were collected at baseline and at 1, 3, and 6 wk of treatment. The experimental protocol was approved by the Human Research Committee of the State University of New York at Buffalo, and each subject signed an informed consent.

Table 1.

<table>
<thead>
<tr>
<th>Marker/wk</th>
<th>Group</th>
<th>0</th>
<th>1</th>
<th>1</th>
<th>3</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>Placebo</td>
<td>80.6 ± 4.1</td>
<td>79.6 ± 3.7</td>
<td>81.1 ± 4.7</td>
<td>80.7 ± 4.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCE</td>
<td>83.1 ± 3.3</td>
<td>84.6 ± 3.2</td>
<td>84.1 ± 2.6</td>
<td>85.3 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>Insulin (μIU/ml)</td>
<td>Placebo</td>
<td>53.5 ± 0.5</td>
<td>5.5 ± 0.6</td>
<td>5.1 ± 0.5</td>
<td>5.0 ± 0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCE</td>
<td>4.7 ± 0.4</td>
<td>4.1 ± 0.4</td>
<td>4.7 ± 0.7</td>
<td>4.5 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>Placebo</td>
<td>0.95 ± 0.21</td>
<td>0.90 ± 0.20</td>
<td>0.94 ± 0.22</td>
<td>0.92 ± 0.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCE</td>
<td>0.98 ± 0.22</td>
<td>0.94 ± 0.24</td>
<td>0.97 ± 0.30</td>
<td>0.93 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>FFA (mM)</td>
<td>Placebo</td>
<td>0.31 ± 0.04</td>
<td>0.32 ± 0.03</td>
<td>0.33 ± 0.03</td>
<td>0.32 ± 0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCE</td>
<td>0.25 ± 0.03</td>
<td>0.29 ± 0.03</td>
<td>0.26 ± 0.03</td>
<td>0.26 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>Placebo</td>
<td>82 ± 15</td>
<td>84 ± 19</td>
<td>87 ± 20</td>
<td>84 ± 18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCE</td>
<td>78 ± 17</td>
<td>77 ± 18</td>
<td>81 ± 18</td>
<td>80 ± 17</td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>Placebo</td>
<td>153 ± 13</td>
<td>159 ± 13</td>
<td>155 ± 12</td>
<td>158 ± 14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCE</td>
<td>159 ± 14</td>
<td>161 ± 12</td>
<td>156 ± 12</td>
<td>159 ± 14</td>
<td></td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>Placebo</td>
<td>46 ± 7</td>
<td>45 ± 7</td>
<td>47 ± 6</td>
<td>47 ± 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCE</td>
<td>45 ± 6</td>
<td>44 ± 6</td>
<td>45 ± 7</td>
<td>45 ± 6</td>
<td></td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>Placebo</td>
<td>93 ± 12</td>
<td>101 ± 14</td>
<td>103 ± 13</td>
<td>98 ± 12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCE</td>
<td>104 ± 15</td>
<td>106 ± 16</td>
<td>98 ± 14</td>
<td>108 ± 15</td>
<td></td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>Placebo</td>
<td>8.32 ± 1.12</td>
<td>8.51 ± 1.21</td>
<td>8.27 ± 1.14</td>
<td>8.38 ± 1.19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCE</td>
<td>8.75 ± 1.07</td>
<td>8.49 ± 1.02</td>
<td>8.59 ± 1.21</td>
<td>8.61 ± 1.25</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± se. No significant changes were observed in these parameters. LDL, Low-density lipoprotein; HDL, high-density lipoprotein.
Nuclear factor-κB (NFκB) DNA binding activity
Nuclear NFκB DNA binding activity was measured by EMSA. Nuclear extracts were prepared from MNCs and by high-salt extraction. The specificity of the bands was confirmed by super shifting these bands with specific antibodies against Rel-A (p65) and p50 (Santa Cruz Biotechnology) and by competition with cold oligonucleotides.

Total RNA isolation and real-time RT-PCR
Total RNA was isolated using commercially available RNAqueous-4PCR kit (Ambion, Austin, TX). Real-time RT-PCR [intraassay coefficient of variation (CV) of 5–8%, interassay CV of 8–12%] was performed using Cepheid Smart Cycler (Sunnyvale, CA), Sybergreen master mix (QIAGEN, Valencia, CA), and gene expression of IKK, JNK, SOCS, PTP, IRS, TLR, IL6, IL-1, and TNF-α mRNA was measured using specific primers (Invitrogen, Carlsbad, CA). The specificity and size of the PCR products were tested by adding a melt curve at the end of the amplifications and running it on a 2% agarose gel. All values were normalized to expression of three housekeeping genes (β-actin, ubiquitin C, and cyclophilin A).

Plasma measurements
Insulin concentrations (intraassay CV of 2.6%, interassay CV of 6.2%) were measured from plasma samples using an ELISA kit (Diagnostics Systems Laboratories, Inc., Webster, TX). Free fatty acid (FFA) concentrations (intraassay CV of 4.6%, interassay CV of 9.2%) were measured using the Half-Micro calorimetric kit (Roche Diagnostic, Indianapolis, IN). Leptin and TNF-α concentrations (intraassay CV of 3.1 and 8%, interassay CV of 5.4 and 10.6%, respectively) were measured from serum by an immunosassay kit from R&D Systems (Minneapolis, MN). C-reactive protein (CRP; intraassay CV of 3%, interassay CV of 3–6%) was measured by immunoassay kit from American Diagnostica Inc. (Stamford, CT).

Statistical analysis
Statistical analysis was conducted using SigmaStat software version 3.1 (SPSS Inc., Chicago, IL). Sample size was calculated based on expected difference of 30% between the groups in NFκB DNA binding as observed in our previous studies (21, 22) with a SD of 20% and desired power of 80%. Data are represented as mean ± SE. Percent change from baseline was calculated and statistical analysis for change from baseline was carried out using one-way, repeated-measures ANOVA (RMANOVA) followed by Holm-Sidak post hoc test. Two-factor RMANOVA analysis followed by Tukey’s post hoc test was used for all multiple comparisons between the two treatment groups.

Results
Effect of PCE on plasma glucose, insulin, leptin, and lipid concentrations
The intake of the extract for 6 wk in normal subjects did not alter plasma insulin or glucose concentrations, nor did it alter homeostatic model on insulin resistance index (HOMA-IR). There was no significant change in plasma FFAs, triglycerides, low-density lipoprotein, high-density lipoprotein, cholesterol, or leptin concentrations after either treatment (Table 1). Serum creatinine and transaminase levels did not alter.

Effect of PCE on ROS generation and P47phox expression by MNCs
ROS generation by MNCs fell significantly after the intake of the extract by 19 ± 10% (P < 0.05, Fig. 1A) at wk 3 and remained suppressed for the 6-wk treatment period, whereas ROS generation did not change in the placebo group. PCE also suppressed p47phox (nicotinamide adenine dinucleotide phosphate oxidase subunit) protein in MNCs by 15 ± 8% below the baseline at 3 wk (P < 0.05, Fig. 1, B

FIG. 1. Change from baseline (%) in ROS generation by MNC (A) and p47 subunit protein (B, C) in MNC following PCE (200 mg/d) containing resveratrol (40 mg/d) or placebo treatment for 6 wk in 10 normal healthy subjects per group. ∗, P < 0.05 comparing changes from baseline by RMANOVA; #, P < 0.05 comparing treatments between the groups by two-way RMANOVA.
and C) and remained below baseline levels at wk 6, whereas it did not change in the placebo group. The changes in ROS generation and p47phox were significantly different between the PCE and placebo groups (two-way RMANOVA).

**Effects of PCE on NFκB DNA binding and proinflammatory mediators**

Intranuclear NFκB DNA binding in MNCs fell significantly by 25 ± 7% below the baseline (P < 0.05, Fig. 2A) at 3 wk of treatment with PCE and remained suppressed thereafter, whereas it did not change significantly in the placebo group. TNF-α and IL-6 mRNA expression in MNCs also fell significantly by 20 ± 7 and 22 ± 9% below the baseline, respectively, at wk 3 (P < 0.05, Fig. 2B and C) after PCE, whereas IL-1β expression did not alter. There was no significant change in any of these indices in the placebo group. The changes in NFκB DNA binding, TNF-α, and IL-6 mRNA expression were significantly dif-

**FIG. 2.** Change from baseline (%) in NFκB DNA binding by EMSA in MNC (A), TNF-α (B), IL-6 (C) mRNA expression in MNC, TNF-α plasma concentrations (D), and CRP concentrations (E) following PCE (200 mg/d) containing resveratrol (40 mg/d) or placebo treatment for 6 wk in 10 normal healthy subjects per group. *, P < 0.05 comparing changes from baseline by RMANOVA; #, P < 0.05 comparing treatments between the groups by two-way RMANOVA.
ferent between the PCE and placebo groups (two-way RMANOVA).

**Effects of PCE on plasma levels of TNF-α and CRP concentrations**

PCE intake also resulted in a significant fall by 33 ± 5% below the baseline (from 0.72 ± 0.2 to 0.46 ± 0.2 pg/ml at wk 3, *P* < 0.05, Fig. 2D) in plasma TNF-α concentrations and by 29 ± 11% below the baseline (from 0.77 ± 0.18 to 0.46 ± 0.1 mg/liter by wk 3, *P* < 0.05, Fig. 2E) in CRP concentrations, whereas there was no change in these mediators in the placebo group. The fall in TNF-α and CRP concentrations was significantly different between the PCE and placebo groups (two-way RMANOVA).

**Effects of PCE on JNK-1, IKKβ, PTP-1B, SOCS-3, IRS-1, and TLR-4 expression in MNCs**

The intake of the extract significantly suppressed *JNK-1*, *IKKβ*, *PTP-1B*, *SOCS-3*, *IRS-1*, and *TLR-4* expression in MNCs.

![Graphs showing the effects of PCE on gene expression](image-url)
This was associated with a significant fall in JNK-1 and PTP-1B proteins by 33/9 and 29/10%, respectively (P < 0.05, Fig. 4, A–C) but not in IKKβ and SOCS-3. On the other hand, PCE-containing resveratrol intake induced a significant increase in the expression of IRS-1 starting at wk 1, reaching 43 ± 8% above the baseline at 3 wk and remaining elevated for the entire treatment period of 6 wk (P < 0.05, Fig. 3E). IRS-1 protein was not detectable in MNCs despite the increase in mRNA levels. There was no change in TLR-4 expression mRNA or protein expression in both groups (data not shown). There was no significant change in any of these genes in the placebo group. Changes in JNK-1, IKKβ, PTP-1B, SOCS-3, and IRS-1 expression after PCE were significantly different when compared with placebo group (two-way RMANOVA).

**Effects of PCE on SIRT-1 expression in MNCs**

There was no significant change in SIRT-1 protein expression in MNCs during this period in both groups.

**Discussion**

Our data show clearly that an extract of *P. cuspidatum*-containing resveratrol suppressed ROS generation by MNCs and the expression of p47phox, the cardinal subunit of nicotinamide adenine dinucleotide phosphate oxidase, the enzyme that converts molecular O₂ into the O₂⁻ radical. In addition, the intranuclear binding of NFkB was also suppressed. NFkB is the major proinflammatory transcription factor. Thus, this extract exerts ROS suppressive (antioxidant) and antiinflammatory effects. These actions, demonstrated for the first time, *in vivo*, are consistent with potential antiatherogenic and antiaging effects. Longer-term studies are required to determine whether these effects are durable and whether higher doses will produce a greater effect.

In addition, there was a significant reduction in the expression of TNF-α and IL-6 in MNCs and the plasma concentration of TNF-α. TNF-α and IL-6 are two major proinflammatory cytokines regulated by NFkB. Plasma CRP concentrations also fell significantly, consistent with an antiinflammatory effect. The magnitude of these effects was similar to that described by us previously for rosiglitazone (22, 23).

In parallel with these effects, the expression of two major proinflammatory kinases, JNK-1 and IKKβ, was also suppressed significantly over the 6-wk period of PCE intake. JNK-1 phosphorylates c-Jun and activates the proinflammatory transcription factor activator protein-1. IKKβ phosphorylates NFkB inhibitor α and thus causes its ubiquitination and proteasomal lysis and the translocation of NFkB into the nucleus and the initiation of proinflammatory transcription. The suppression of these kinases is thus antiinflammatory. In addition, there was also a reduction in the expression of SOCS-3, a protein whose expression is modulated by the proinflammatory cytokines TNF-α, IL-1β, and IL-6 (24, 25) and has been shown to be up-regulated in obesity (26). PCE-containing resveratrol also caused suppression in the expression of PTP-1B that, in addition to its known negative effects on insulin action, has been shown to be involved in the in-
flammatory response (17). These observations further support a potent antiinflammatory effect of PCE extract in vivo. It is also noteworthy that the suppression of PTP-1B was not associated with a change in the expression of SIRT-1 because previous work in animal models showed that resveratrol-induced suppression of PTP-1B was mediated by SIRT-1 (15).

On the other hand, TLR4 expression was not altered significantly. TLR4 is the specific receptor for endotoxin or lipopolysaccharide, which triggers the downstream responses leading activation of NFκB.

It is of interest that several of the proinflammatory genes suppressed by the resveratrol-containing extract also interfere with insulin signal transduction and may play a role in the development of insulin resistance. The two kinases, IKKβ and JNK-1, cause serine phosphorylation of IRS-1 and thus interfere with insulin signal transduction (27). SOCS-3, which has been previously shown to be related to body mass index and HOMA-IR and inversely to insulin receptor phosphorylation in obese humans (26), also interferes with insulin signal transduction by causing the ubiquitination and proteasomal degradation of IRS-1 and IRS-2 (28). PTP-1B is the phosphatase that removes phosphate residues from phosphorysine in the β-subunit of the insulin receptor and thus limits the magnitude and the duration of the insulin signal at the insulin receptor level (18). In addition, the specific individual deletions of SOCS-3, JNK-1, IKKβ, TLR-4, and PTP-1B protect against the development of diet-induced, obesity-related insulin resistance (3, 27, 29–31). In view of the above observations, it is possible that PCE with resveratrol may be a potential insulin sensitizer because it suppressed SOCS-3, JNK-1, IKKβ, and PTP-1B. Consistent with this effect, there was also an increase in the expression of IRS-1. Clearly this hypothesis needs to be tested in an insulin-resistant population.

The major weakness of our study is that it does not identify which component of the extract is responsible for the effects observed. The PCE used has only 20% resveratrol and the effects that we described may be due to products other than resveratrol contained in that preparation. This was the purest preparation available more than 2 yr ago when these experiments were conducted. Purer preparations are available now and would need to be tested. On the other hand, our observations also open the way for the investigation of other constituents of *P. cuspidatum*, which may be responsible for these very potent and interesting biological and clinically relevant effects. There are data showing the antiinflammatory effects of polyphenols, in vitro, but there are no data demonstrating this, in vivo, in the human (32). The other limitation of this study is that it has been conducted in a relatively small number of patients and for a short period of 6 wk.

In conclusion, an extract of *P. cuspidatum* suppresses ROS generation by MNCs; the expression of p47phox; the intranuclear binding of NFκB; the expression of TNF-α, IL-6, SOCS-3; and plasma concentrations of TNF-α and CRP. It also suppresses the expression of JNK-1 and IKKβ, both of which are mediators of inflammation. In addition, it suppresses the expression of SOCS-3 and PTP-1B, both of which are known to interfere with insulin signal transduction. This would potentially prolong insulin action and its antiinflammatory effects. These comprehensive suppressive effects on ROS generation and inflammation are consistent with an antiaging action of resveratrol.

Acknowledgments

Address all correspondence and requests for reprints to: Paresh Dandona, B.Sc., M.B.B.S., D.Phil., F.R.C.P., Director, Diabetes-Endocrinology Center of Western New York, Chief of Endocrinology, State University of New York at Buffalo, 3 Gates Circle, Buffalo, NY 14209. E-mail: pdandona@kaleidahealth.org.

P.D. is supported by National Institutes of Health Grants R01DK069805-02 and R01DK075877-01-A2 and American Diabetes Association Grant 08-CR-13.

Disclosure Summary: The authors have nothing to disclose.

References


Effect of DHA-rich fish oil on PPARγ target genes related to lipid metabolism in type 2 diabetes: A randomized, double-blind, placebo-controlled clinical trial. J Clin Lipidol. 2015


Randomized, crossover, head-to-head comparison of EPA and DHA supplementation to reduce inflammation markers in men and women: the Comparing EPA to DHA Study.
Randomized, crossover, head-to-head comparison of EPA and DHA supplementation to reduce inflammation markers in men and women: the Comparing EPA to DHA Study1–3

Janie Allaire,4 Patrick Couture,4,5 Myriam Leclerc,4 Amélie Charest,4 Johanne Marin,4 Marie-Claude Lépine,4 Denis Talbot,6 Andrée Tchernof,4,5,7 and Benoît Lamarche4,*

4Institute of Nutrition and Functional Foods, Pavillon des Services, 5University Hospital Center (CHU) of Quebec Research Center, and 6Department of Social and Preventive Medicine, Laval University, Quebec, Canada; and 7Quebec Heart and Lung Institute, Quebec, Canada

ABSTRACT

Background: To date, most studies on the anti-inflammatory effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in humans have used a mixture of the 2 fatty acids in various forms and proportions.

Objectives: We compared the effects of EPA supplementation with those of DHA supplementation (re-esterified triacylglycerol; 90% pure) on inflammation markers (primary outcome) and blood lipids (secondary outcome) in men and women at risk of cardiovascular disease.

Design: In a double-blind, randomized, crossover, controlled study, healthy men (n = 48) and women (n = 106) with abdominal obesity and low-grade systemic inflammation consumed 3 g/d of the following supplements for periods of 10 wk: 1) EPA (2.7 g/d), 2) DHA (2.7 g/d), and 3) corn oil as a control with each supplementation separated by a 9-wk washout period. Primary analyses assessed the difference in cardiometabolic outcomes between EPA and DHA.

Results: Supplementation with EPA compared with supplementation with EPA led to a greater reduction in interleukin-18 (IL-18) (−7.0% ± 2.8% compared with −0.5% ± 3.0%, respectively; P = 0.01) and a greater increase in adiponectin (3.1% ± 1.6% compared with −1.2% ± 1.7%, respectively; P < 0.001). Between DHA and EPA, changes in CRP (−7.9% ± 5.0% compared with −1.8% ± 6.5%, respectively; P = 0.25), IL-6 (−12.0% ± 7.0% compared with −13.4% ± 7.0%, respectively; P = 0.86), and tumor necrosis factor-α (−14.8% ± 5.1% compared with −7.6% ± 10.2%, respectively; P = 0.63) were NS. DHA compared with EPA led to more pronounced reductions in triglycerides (−13.3% ± 2.3% compared with −11.9% ± 2.2%, respectively; P = 0.005) and the cholesterol:HDLC cholesterol ratio (−2.5% ± 1.3% compared with −0.7% ± 1.1%, respectively; P = 0.006) and greater increases in HDL cholesterol (7.6% ± 1.4% compared with −0.7% ± 1.1%, respectively; P < 0.0001) and LDL cholesterol (6.9% ± 1.8% compared with 2.2% ± 1.6%, respectively; P = 0.04). The increase in LDL-cholesterol concentrations for DHA compared with EPA was significant in men but not in women (P-treatment × sex interaction = 0.046).

Conclusions: DHA is more effective than EPA in modulating specific markers of inflammation as well as blood lipids. Additional studies are needed to determine the effect of a long-term DHA supplementation per se on cardiovascular disease risk. This trial was registered at clinicaltrials.gov as NCT01810003. Am J Clin Nutr doi: 10.3945/ajcn.116.131896.

Keywords: DHA, EPA, inflammation, men and women, randomized controlled trial, risk factors

INTRODUCTION

Subclinical inflammation is recognized as a key etiologic factor in the development of atherosclerosis that leads to ischemic heart disease (IHD).8 (1, 2). There is a growing body of literature that has suggested that long-chain ω-3 (n–3) PUFA (LCn–3PUFAs), primarily EPA (20:5n–3) and DHA (22:6n–3), may attenuate the proinflammatory state that is associated with obesity and metabolic syndrome (MetS) (3). In that regard, a number of mechanisms supporting the purported anti-inflammatory effects of LCn–3PUFAs have been proposed. These mechanisms include the inhibition of the proinflammatory nuclear transcription factor NFκB in various

1Supported by the Canadian Institutes for Health Research (CIHR) (grant MOP-123494; to PC, AT, and BL). JA is a recipient of a PhD Scholarship from the CIHR and the Fonds de recherche du Québec - Santé. Douglas Laboratories provided the EPA, DHA, and control capsules used in this study. This is a free access article, distributed under terms (http://www.nutrition.org/publications/guidelines-and-policies/license/) that permit unrestricted noncommercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

2The Canadian Institutes for Health Research was not involved in designing the study; conducting the study; the collection, management, analysis, or interpretation of the data; or preparation and review of the manuscript before submission.

3Supplemental Tables 1–6 are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

*To whom correspondence should be addressed. E-mail: benoit.lamarche@fsaa.ulaval.ca.

8Abbreviations used: apoB, apolipoprotein B; CRP, C-reactive protein; CVD, cardiovascular disease; IHD, ischemic heart disease; ITT, intent to treat; LCn–3PUFA, long chain ω-3 (n–3) PUFA; MetS, metabolic syndrome; RCT, randomized controlled trial.

Received February 1, 2016. Accepted for publication May 2, 2016. doi: 10.3945/ajcn.116.131896.
tissues through a series of metabolic cascades involving the activation of peroxisome proliferator activated receptor-γ and several other signaling proteins (4).

A recent meta-analysis of randomized controlled trials (RCTs) substantiated the anti-inflammatory effect of LCn-3PUFA supplementation as evidenced by significant reductions in plasma C-reactive protein (CRP), IL-6, and TNF-α concentrations (5). This analysis was based on data from 68 RCTs and 4601 individuals with or without chronic nonautoimmune diseases such as dyslipidemia, obesity, type 2 diabetes, and MetS. The meta-analysis revealed significant research gaps pertaining to the effects of LCn-3PUFAs on inflammation markers. Most importantly, almost all of the RCTs available thus far have either used a mix of EPA and DHA in various ratios or have investigated only one of the 2 LCn-3PUFAs. The use of these methods is not a trivial issue considering that DHA appears to be more potent than is EPA in modulating plasma lipid concentrations (6). Therefore, it remains largely unknown whether EPA and DHA have similar or different effects on markers of inflammation. Other significant shortcomings have included the fact that almost all of the available RCTs to date were not designed a priori to investigate the effect of EPA or DHA on markers of subclinical inflammation as a primary outcome and were also based on sample sizes that may have been too small to yield robust results. Finally, whether sex influences the efficacy of EPA and DHA to modulate markers of inflammation is an area of great interest that remains speculative. Addressing these gaps has important public health implications considering that EPA plus DHA supplements are broadly recommended by various health agencies, including the American Heart Association (7), for IHD risk prevention or the management of triglycerides.

The Comparing EPA to DHA Study is a double-blind, randomized, crossover, controlled study that was specifically designed to compare the effects of EPA and DHA on inflammation markers in individuals with abdominal obesity and subclinical inflammation. As a secondary objective, we compared the effects of EPA and DHA on plasma lipids and verified if responses to EPA and DHA in men and women are similar. We hypothesized that DHA is more potent than is EPA in modulating inflammatory markers and plasma lipid concentrations. However, on the basis of evidence that suggested that platelet aggregation is more responsive to EPA in men and to DHA in women (8), we also hypothesized that EPA supplementation induces a greater anti-inflammatory response than does DHA in men, whereas women are more responsive to supplementation with DHA.

**METHODS**

**Study design**

This study used a double-blind, randomized, controlled, crossover design with 3 treatment phases as follows: 1) EPA, 2) DHA, and 3) corn oil as a control. Each treatment phase had a median duration of 10 wk. The median washout time between treatments was 9 wk. The random assignment of participants to one of 6 treatment sequences was performed with the use of an inhouse computer program and was stratified by sex. Allocations to treatments were concealed from participants, study coordinators, and laboratory technicians throughout the study. Codes were unconcealed after all primary statistical analyses had been completed. Participants were supplemented with 3 identical 1-g capsules of >90% fish oils/d that provided 2.7 g EPA/d, 2.7 g DHA/d, and 0 g EPA and DHA/d (3 g corn oil was used as the control). Supplements were formulated as re-esterified triacylglycerol and provided by Douglas Laboratories. Participants were instructed to maintain a constant body weight during the course of the study. Subjects were also counseled about how to exclude fatty fish (including salmon, tuna, mackerel, and herring), fish-oil supplements, flax products, walnuts, and ω-3–enriched products during the 3 study phases. Vitamin supplements and natural health products were allowed at a stable dose. Alcohol consumption was permitted during the study of intakes that did not exceed 1 or 2 servings alcohol/d (12–15 g alcohol/d) but was forbidden during the 4 d that preceded blood draws. Subjects were also instructed to maintain their usual physical activity except during the 4 d that preceded blood sampling at the various stages of the study during which they were asked not to engage in any form of vigorous physical activity.

**Study population**

The a priori–defined eligibility criterion was to have MetS as per the International Diabetes Federation definition (9). However, this criterion was modified 2 mo into recruitment because of unforeseen difficulties in achieving the intended sample size with the use of such a criterion (eligibility rate was 2.4% on the basis of 170 screens). Eligibility criteria were modified to include having abdominal obesity per International Diabetes Federation sex-specific cutoffs (≥80 cm for women; ≥94 cm for men) (9) in combination with a screening plasma CRP concentration >1 but <10 mg/L. Subjects had to be otherwise healthy. These new criteria were consistent with the primary aim of the study, which was to compare the effects of EPA and DHA supplementation on markers of inflammation. Subjects were recruited at the Institute of Nutrition and Functional Foods via the media (newspaper and radio) and electronic newsletters. Subjects had to be aged between 18 and 70 y and have stable body weight for ≥3 mo before random assignment. In premenopausal women, only those individuals with a regular menstrual cycle (25–35 d) for the past 3 mo were included. Follicle-stimulating hormone measurements were performed when needed to confirm the premenopausal status (follicle-stimulating hormone concentration <25 IU/L) (10). Women who were using contraceptive agents were eligible. The use of contraceptive agents was documented and adjusted for if required (see Statistical analyses section). Evidence has suggested that phases of the menstrual cycle have little effects on markers of inflammation (11), and therefore, collections of samples were not adjusted for the menstrual cycle. Exclusion criteria were a plasma CRP concentration >10 mg/L at screening, extreme dyslipidemias such as familial hypercholesterolemia, having a personal history of cardiovascular diseases (IHD, cerebrovascular disease, or peripheral arterial disease), taking medications or substances known to affect inflammation (e.g., taking steroids or binging alcohol), and the use of LCn-3PUFA supplements ≤2 mo of study onset. Postmenopausal women who were receiving hormone replacement therapy at a stable dose were included (12). All participants signed an informed consent document that was approved by the local ethics committees at the beginning of the study, and the study protocol was registered at clinicaltrials.gov (NCT01810003) on 4 March 2013.
Anthropometric measures

Anthropometric measures, including waist and hip circumferences, were obtained according to standardized procedures (13). Body composition was measured with the use of dual-energy X-ray absorptiometry (GE Healthcare).

Risk factor assessment

Plasma CRP concentrations were measured with the use of the Behring Latex-Enhanced highly sensitive assay on the Behring Nephelometer BN-100 system (Behring Diagnostic) and the calibrators (N Rheumatology Standards SL) provided by the manufacturer as described previously (14). Other inflammation markers were measured with the use of commercial ELISA kits for the human form of the cytokine as follows: IL-6 and TNF-α (HS600B and HSTA00D; R&D Systems), IL-18 (7620; MBL International), and adiponectin (K1001-1; B-Bridge International). Serum total cholesterol, triglycerides, and HDL cholesterol were assessed on a Roche/Hitachi Modular system (Roche Diagnostics) according to the manufacturer’s specifications and with the use of proprietary reagents. Plasma LDL-cholesterol concentrations were calculated with the use of Friedewald’s equation. Total plasma apolipoprotein B (apoB) concentrations were measures with the use of a commercial ELISA kit (A70102; Alerchek Inc.). CVs for each analyte are shown in Supplemental Table 1. Total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, apoB, CRP, IL-6, and adiponectin were measured twice on consecutive days at the end of each treatment to reduce variation and, hence, increase statistical power. The mean of the 2 measurements were used in the analyses. Analyses for TNF-α and IL-18 were based on a single measure posttreatment. Treatment-specific baseline values were measured once. All personnel involved in the measurements of study outcomes were blinded to treatments.

Compliance

Compliance to supplementation was assessed by counting supplements that were returned to study coordinators by participants. Subjects with compliance <80% during any given treatment were considered to be noncompliant, and data from that particular treatment were excluded from analyses. EPA and DHA contents in plasma phospholipids were also used as another proxy of compliance in a randomly selected subsample of participants (n = 30; 15 women and 15 men). EPA and DHA contents of plasma phospholipids were measured before and after treatments as described previously (15). The fatty acid composition of plasma phospholipids was expressed as a percentage of all fatty acids. The concurrent use of a medication during the experimental protocol was tracked with the use of checklists. Participants were asked to notify the physician in charge of the clinical aspects of the study before the initiation of any medication. Variations in dietary habits during the intervention were monitored with the use of a validated quantitative web-based food-frequency questionnaire at the end of each treatment phase (16). Usual physical activity was monitored with the use of a 3-d validated physical activity journal (17).

Sample-size calculation

A priori sample-size calculations indicated that n = 150 individuals would allow us to detect a minimal difference of 10% in plasma CRP concentrations when any 2 treatments were compared with a power of 81% and P < 0.01 (2 tailed) (18). CRP was used as the primary outcome measure for sample-size calculations because it is considered a key variable for the assessment of the inflammatory status in clinical practice (19). A 10% reduction in plasma CRP was considered to be of clinical relevance on the basis of several epidemiologic studies that have shown a linear relation between CRP and risk of IHD (19). The power to detect a significant treatment × sex interaction was estimated with the use of the GLMPower procedure in SAS software (v9.3; SAS Institute Inc.) with treatment, sex, and treatment × sex as main effects. On the basis of SD estimates (35%) and with consideration of a sample size of 150 individuals equally distributed between men and women, the power was 80% to detect a significant treatment × sex interaction (P < 0.05) for a reduction in plasma CRP, compared with the control value, that was ≥10% with treatment A (no change with treatment B) in men and ≥10% with treatment B (no change with treatment A) in women. The minimal detectable difference between treatments in plasma CRP within each sex was 11.5% [power: 80%; P <0.05 (2-tailed); with n = 75 in each group]. The anticipated sample size provided high statistical power to investigate changes in lipid concentrations (not shown).

Statistical analyses

Differences in study outcomes between treatments were assessed with the use of the MIXED procedure for repeated measures in the SAS program (v9.4) with treatment, sex, and the treatment × sex interaction (when significant) as fixed effects and a compound symmetry or autoregressive covariance matrix to account for within-subject correlations. The change of each treatment compared with the control value (posttreatment EPA minus control and DHA minus control) was used as the dependent variable in all analyses as per our a priori–defined analytic plan (20). To be included in the analyses, subjects had to have completed the control phase plus ≥1 of the 2 treatment phases. With this approach, the main treatment effect in the mixed models reflected the direct effect of each treatment phase plus comparison of EPA and DHA and was considered the primary main treatment effect in the mixed models. With this approach, the main treatment effect had only 2 levels. In the same model and as secondary analyses, the change for each treatment compared with the control value (posttreatment EPA minus control and DHA minus control) was used as the dependent variable in all analyses as per our a priori–defined analytic plan (20). To be included in the analyses, subjects had to have completed the control phase plus 1 of the 2 treatment phases. With this approach, the main treatment effect in the mixed models reflected the direct comparison of EPA and DHA and was considered the primary analysis. Adjustment for multiple comparisons was not necessary because the main treatment effect had only 2 levels. In the same model and as secondary analyses, the change for each treatment compared with the control value was tested against the null hypothesis by the LSMEANS statement in the MIXED procedure. Potential confounders of the outcome measure response to treatment, mainly obesity and body fat status, age, use of contraceptive agents (premenopausal women), menopausal status, energy and nutrient intakes, and the sequence of treatments, were considered by integrating interaction terms with the main treatment effect into the mixed models. Results from analyses that were based on the most parsimonious models (i.e., retaining only the variables that contributed significantly to variations in any given study outcome) are shown. All primary statistical analyses were undertaken in a blinded fashion with the use of study codes for each treatment. The skewness in the distribution of all study outcomes was considered, and data were transformed when required. In the case of the CRP analysis per se, missing values were attributed when the mean of the 2 consecutive posttreatment CRP values was >10 mg/L. Because the MIXED procedure is robust to missing data, analyses were first conducted without the multiple imputation of missing data (21). However,
we also analyzed the study results with the use of an intent-to-treat (ITT) approach with the multiple imputation of missing data. Comparisons between treatments (significant compared with NS) were unchanged in the ITT analysis with only slightly larger P values with the ITT approach for the significance of the treatment effect for IL-18 and adiponectin. Details are provided in Supplemental Table 2.

RESULTS

Baseline characteristics of subjects

Figure 1 represents the Consolidated Standards of Reporting Trials flow diagram of the study (22), which was initiated on 3 April 2013 and was completed on 19 June 2015. Of 173 eligible men and women, a total of 154 subjects were randomly assigned to treatment sequences. The dropout rate was 20% (n = 31 of 154 randomly assigned participants). Treatment-specific data of participants with compliance <80% (n = 2 for the EPA treatment compared with the control) were excluded from analyses. Characteristics at the screening visit of the 154 subjects randomly assigned into the study are shown by sex (n = 48 for men; n = 106 for women) in Table 1. Sixty-six percent of women were postmenopausal, of whom 23% were receiving hormone therapy. Seventy-two percent of premenopausal women were using contraceptive agents. As per the inclusion criteria, all subjects had a high waist circumference (≥94 cm for men and ≥80 cm for women) and an elevated plasma CRP concentration as a group but were otherwise healthy. There was no difference in the baseline characteristics of participants between treatments (Supplemental Table 3). We showed marginal differences concerning dietary intakes of fibers, proteins, EPA, and DHA as well as a significant difference in alcohol consumption between treatments (Supplemental Table 4). However, such differences in nutrient intakes had no effect on study outcomes (P > 0.05) and thus were not included in the final mixed models.

Compliance to treatments and side effects

The mean compliance to supplementation during each treatment phase that was based on returned capsules was high (control: 97% ± 5%; EPA: 97% ± 6%; DHA: 96% ± 5%) and not significantly different between treatments (Kruskal-Wallis test: P = 0.17). The plasma phospholipid fatty acid composition that was measured posttreatment is presented in Supplemental Table 5. The phospholipid fatty acid profile tracked well with each supplementation phases, which also reflected a high compliance with treatments. Changes in plasma phospholipid concentrations of EPA, docosapentaenoic acid, and DHA after each treatment were similar between men and women with the exception of plasma docosapentaenoic acid concentrations, which were higher in men than in women after EPA supplementation (2.56% ± 0.11% vs. 2.43% ± 0.12% in men and women, respectively).

TABLE 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Men (n = 48)</th>
<th>Women (n = 106)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>57 ± 12</td>
<td>50 ± 16</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>30 ± 4</td>
<td>29 ± 4</td>
</tr>
<tr>
<td>Waist, cm</td>
<td>107 ± 10</td>
<td>98 ± 9</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>119 ± 13</td>
<td>111 ± 12</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>78 ± 9</td>
<td>71 ± 8</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.9 ± 0.7</td>
<td>5.5 ± 0.9</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.0 ± 0.7</td>
<td>3.2 ± 0.8</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.3 ± 0.3</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>Cholesterol:HDL-cholesterol ratio</td>
<td>4.0 ± 0.9</td>
<td>3.5 ± 1.0</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.5 ± 0.8</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>2.8 ± 1.8</td>
<td>3.7 ± 2.4</td>
</tr>
<tr>
<td>Fasting glucose, mmol/L</td>
<td>5.5 ± 0.9</td>
<td>5.2 ± 0.7</td>
</tr>
<tr>
<td>Subjects with MetS, n (%)</td>
<td>25 (12)</td>
<td>17 (19)</td>
</tr>
</tbody>
</table>

CRP, C-reactive protein; DBP, diastolic blood pressure; SBP, systolic blood pressure; MetS, metabolic syndrome.

Mean ± SD (all such values).

n = 47 in men because of a missing value.
Changes compared with control values in posttreatment inflammation markers and blood lipids with EPA and DHA

**TABLE 2**

<table>
<thead>
<tr>
<th>Inflammation markers</th>
<th>Control</th>
<th>ΔEPA compared with control</th>
<th>ΔDHA compared with control</th>
<th>P&lt;ΔEPA compared with ΔDHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP, mg/L</td>
<td>3.02 ± 0.14</td>
<td>-0.05 ± 0.14</td>
<td>0.45</td>
<td>-0.23 ± 0.14</td>
</tr>
<tr>
<td>IL-6, pmol/L</td>
<td>1.61 ± 0.16</td>
<td>-0.21 ± 0.10</td>
<td>0.03</td>
<td>-0.19 ± 0.10</td>
</tr>
<tr>
<td>IL-18, pmol/L</td>
<td>271.7 ± 12.6</td>
<td>-2.12 ± 6.29</td>
<td>0.38</td>
<td>-18.15 ± 6.25</td>
</tr>
<tr>
<td>TNF-α, pmol/L</td>
<td>1.35 ± 0.14</td>
<td>-0.11 ± 0.10</td>
<td>0.10</td>
<td>-0.20 ± 0.05</td>
</tr>
<tr>
<td>Adiponectin, mg/L</td>
<td>7.03 ± 0.46</td>
<td>-0.08 ± 0.12</td>
<td>0.14</td>
<td>0.22 ± 0.12</td>
</tr>
</tbody>
</table>

Blood lipids

| Total cholesterol, mmol/L | 5.16 ± 0.08 | -0.03 ± 0.05 | 0.62 | 0.15 ± 0.05 | 0.001 | <0.001 |
| LDL cholesterol, mmol/L  | 2.99 ± 0.07 | 0.07 ± 0.04 | 0.046 | 0.16 ± 0.04 | <0.0001 | 0.04 |
| HDL cholesterol, mmol/L  | 1.54 ± 0.04 | -0.01 ± 0.02 | 0.48 | 0.11 ± 0.02 | <0.0001 | <0.0001 |
| Cholesterol:HDL cholesterol ratio | 3.55 ± 0.09 | 0.01 ± 0.04 | 0.86 | -0.10 ± 0.05 | <0.0001 | 0.006 |
| apoB, g/L               | 1.31 ± 0.04 | 0.01 ± 0.02 | 0.46 | 0.03 ± 0.02 | 0.02 | 0.16 |
| Triglycerides, mmol/L   | 1.38 ± 0.06 | -0.16 ± 0.03 | <0.0001 | -0.25 ± 0.04 | <0.0001 | 0.005 |

1All values are unadjusted means ± SEMs. n = 125 for the control, n = 121 for the ΔEPA, and n = 123 for the ΔDHA. apoB, apolipoprotein B; CRP, C-reactive protein.
2P values for EPA and DHA changes compared with control values in the outcome were determined with the use of the LSMEANS statement and were tested against the null hypothesis in mixed models (SAS v9.4; SAS Institute Inc.).
3Adjustment for potential covariates (sex, age, weight, waist circumference, menopausal status, value of control treatment, treatment-specific baseline value, and sequence of treatments) was considered only when the covariates were shown to be significant at P < 0.05 in mixed models.
4Main treatment P values for the comparison between EPA and DHA changes compared with control values in the outcome were determined with the use of the main treatment effect in mixed models. The mixed model for the main effect for the comparison of ΔEPA and ΔDHA was based on n = 123 observations with the exclusion of ΔEPA data for 2 participants because of low compliance.
5Log-transformed data were used in these analyses because of the skewness of distributions of posttreatment values.
6n = 117 for the control. Because of exclusions that were due to CRP concentrations >10 mg/L after the treatment phase, n = 110 for EPA changes from control values, and n = 111 for DHA changes from control values.

Changes compared with control values in posttreatment body mass index, waist circumference, and percentages of fat and android fat (data not shown). Table 2 presents absolute changes in study outcomes after EPA and DHA treatment compared with control values, whereas Figure 2 depicts treatment-specific changes in relative terms.

**FIGURE 2** Mean ± SEM percent changes (Δ) compared with control values in posttreatment inflammation markers and blood lipids with EPA and DHA. Mixed models provided P values for main treatment effects (ΔEPA compared with ΔDHA). **Δ**P < 0.05 for within-treatment effects (compared with control values) as shown in Table 2 and determined with the use of the LSMEANS statement in the mixed models. n = 121 for EPA changes from control values, and n = 123 for DHA changes from control values. Apo B, apolipoprotein B; C, cholesterol; CRP, C-reactive protein; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; TG, triglyceride.

**Inflammation markers**

Compared with the EPA treatment, supplementation with DHA led to a greater reduction in serum IL-18 (P = 0.01) and a greater increase in adiponectin (P < 0.001) (Table 2, Figure 2). Changes in serum concentrations of CRP (P = 0.25), IL-6 (P = 0.86), and TNF-α (P = 0.63) were not different between DHA and EPA treatments. Compared with the control, EPA significantly decreased plasma IL-6 concentrations (~13.4%) but had no significant effect on other inflammation markers. Compared with the control, DHA supplementation significantly decreased plasma CRP (~7.9%), IL-6 (~12.0%), IL-18 (~7.0%), and TNF-α (~14.8%) concentrations and increased adiponectin concentrations (~3.1%). There was no significant treatment × sex interaction in the response of inflammation markers to EPA and DHA.

**Blood lipids**

Compared with the EPA treatment, supplementation with DHA reduced triglycerides (P = 0.005), the cholesterol:HDL-cholesterol ratio (P = 0.006), and increased serum concentrations of HDL cholesterol (P < 0.0001) and LDL cholesterol (P = 0.04) (Table 2, Figure 2). There was a treatment × sex interaction (P = 0.0455) in the LDL-cholesterol response to EPA and DHA (Figure 3). DHA supplementation increased LDL cholesterol more than did EPA.
The current study addressed key research gaps pertaining to the effect of LCn–3PUFAs on surrogate markers of inflammation. Previous RCTs on the topic have yielded inconsistent results because of a number of experimental and methodologic factors (5, 23). First and foremost, most of the available RCTs in healthy subjects or in subjects who were at risk of cardiovascular disease (CVD) investigated the effect of LCn–3PUFAs on inflammation markers as a secondary outcome and not as the primary outcome. In the meta-analysis by Li et al. (5), only one study provided a head-to-head comparison of EPA and DHA on inflammation markers in the 68 RCTs reviewed. The study, in patients with type 2 diabetes, showed no significant effect of EPA or DHA supplementation (4 g/d for 6 wk) on CRP, IL-6, and TNF-α concentrations but was based on a sample of only 25 patients/group (24). Two recent studies have compared EPA and DHA directly (<2 g/d) and showed no significant effect on CRP and proinflammatory cytokines (25, 26). These parallel-arm studies comprised <20 subjects/group and, therefore, were also clearly underpowered to yield robust results. These examples emphasize how studies thus far, in almost all cases, were not adequately designed to specifically investigate markers of inflammation. In that context, results from this large RCT provide novel and meaningful information to our knowledge.

Supplementation with DHA (2.7 g/d) for 10 wk decreased serum IL-18 and increased adiponectin significantly more than did supplementation with EPA (2.7 g/d). Also, the reduction in plasma CRP concentrations with DHA compared with control oil was almost 4-fold greater in magnitude than the reduction with EPA although this difference did not reach significance. The data confirm the indirect evidence from the meta-analysis by Li et al. (5), which suggested that the anti-inflammatory effects of mixed LCn–3PUFAs seen in previous studies may have been attributable to DHA. Results from a meta-analysis of 13 RCTs suggested a modest increase in plasma adiponectin concentrations with LCn–3PUFA supplementation (27), but our data indicate that this effect may also be attributable more specifically to DHA.

Mendelian randomization studies have indicated that increased plasma CRP concentrations are unlikely to be even a modest causal factor for CVD (28). This suggestion does not rule out the importance of inflammation in the etiology of atherosclerosis and resulting CVD (2). Data from the Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin trial have shown that the statin treatment of patients with low LDL-cholesterol concentrations but with subclinical inflammation (CRP concentration >2 mg/L) was highly effective in reducing risk of vascular events, myocardial infarction, and stroke (29). Data from the current study indicate that DHA compared with the control corn oil is effective in reducing plasma concentrations of several inflammation markers (IL-6, IL-18, and TNF-α) in addition to CRP. IL-18 is expressed in human atheroma where it influences the expression of adhesion molecules, chemokines, cytokines and matrix metalloproteinases. IL-6 is involved in the acute-phase response by inducing the production of CRP and other inflammatory markers in the liver. The expression of IL-6 is also stimulated in smooth muscle cells by circulating IL-18 and other cytokines (30). Changes in each of these surrogate markers suggest significant effects of DHA on a variety of inflammation processes. EPA enhances the synthesis of the E-series resolvins, whereas DHA leads to the production of the D-series resolvins in addition to enhancing the synthesis of protectins and maresins, all of which may have different anti-inflammatory properties. Although relatively well characterized in cell and animal models (31), the potentially distinct contributions of resolvins from EPA and protectins and maresins from DHA on inflammation processes have not been well characterized in vivo in humans (4, 23).

Although supplementation with a mixture of EPA and DHA may activate the peroxisome proliferator activated receptor-α pathway in both sexes, the nuclear transcription factor κB pathway...
appears to be activated in men only (32), which suggests that men and women may respond differently to EPA and DHA supplement-
ation. Our results are not consistent with these data or with our a priori hypothesis that there is a sex-specific anti-inflammatory response to EPA and DHA.

LCn–3PUFA and plasma lipids

A meta-analysis of RCTs that compared the effect of different doses of EPA and DHA on blood lipids has been published (6). In the 21 studies included in the meta-analysis, 10 studies compared EPA with a control, 17 studies compared DHA with a control, and only 6 studies compared EPA with DHA directly. Results from our own study are consistent with specific analyses of these 6 head-to-head comparison studies of EPA and DHA in showing significantly greater reduction in plasma triglycerides and signi-
ficantly greater increases in plasma LDL cholesterol and HDL cholesterol with DHA than with EPA. The fact that the LDL–cholesterol–raising effect of DHA seems to be more pronounced in men than in women deserves further investigation. We showed that the increase in total plasma apoB after DHA supplementation was one-half that of LDL cholesterol. This result, combined with a greater reduction in serum triglycerides, suggests an increase in the LDL particle size with DHA as well (33). This assumption needs to be verified by proper measures of the change in LDL particle size with EPA and DHA.

This study has several strengths but also some limitations that need to be outlined. To the best of our knowledge, this is the largest crossover-design study to provide a head-to-head compari-
on of the effects of EPA and DHA on inflammatory markers as a primary outcome. The repeated measures after treatment reduced the intraindividual variability of the results and, hence, increased the statistical power. The use of corn oil as a control may have blunted the effects of EPA and DHA on some of the study outcomes. However, many previous RCTs used various veget-
etable oils as a control, and to that extent, our study design was similar to that of previous studies on this topic (5). Compared with baseline values, corn-oil supplementation decreased total cholesterol and LDL-cholesterol concentrations but had no effect on other markers in this study (data not shown). Concentrations of EPA and DHA in plasma phospholipids were measured only posttreatment, and hence, it was not possible to verify that the values had returned to baseline concentrations after each washout period. However, concentrations of blood lipids and inflamma-
tory markers were similar at baseline in the 3 treatments (Sup-
plemental Table 3). There was also no significant sequence-
by-treatment interaction on the study outcomes. These results provide convincing evidence of no residual or carryover effects of a treatment onto the subsequent treatment. The 20% dropout rate remains acceptable for a crossover study of a total duration of 46 wk. Also, the number of subjects who were eligible for statistical analyses was lower than our sample-size target, which implied that detectable effect sizes were slightly larger than anticipated.

Inflammation markers are known to be sensitive to acute immune challenges. However, sensitivity analyses that excluded values greater than the 95th percentile for each risk factor had no effect on the results (not shown), which made the results quite robust. The use of mixed models compared with an ITT approach for the analysis of data from RCTs is a controversial issue. Nevertheless, both methods yielded almost identical results, which supported the robustness of the experimental data.

In conclusion, data from this carefully controlled RCT indicate that DHA supplementation at a dose of ~3 g/d for 10 wk may be more potent in modulating inflammation markers than would be a similar dose of EPA in men and women with abdominal obesity and subclinical systemic inflammation but who are otherwise healthy. To our knowledge, these are impor-
tant new data because most available studies have been un-
dertaken with the use of mixtures of various ratios of EPA and DHA. Consistent with previous studies, DHA was also more potent than EPA in modulating lipid risk factors. The extent to which such differences between EPA and DHA in modulating lipid and inflammation risk factors are meaningful in terms of CVD-risk prevention remains unclear and need to be investigated in the future.

We thank Steeve Larouche and Danielle Aubin at the Institute of Nutrition and Functional Foods for their technical assistance and for the expert care provided to participants. We also thank Pierre Julien and his research team from the Centre Hospitalier Universitaire de Québec Research Center, for the measurement of plasma phospholipid fatty acids.

The authors’ responsibilities were as follows—JA: performed the statistical analyses and wrote the manuscript; PC: was responsible for the screening and medical supervision of the study participants; PC, AT, and BL: designed the research; ML, AC, JM, and MC-L: conducted the research; DT: critically revised the statistical analysis methods of the manuscript and provided feedback on the overall content of the manuscript; BL: had primary responsibility for the final content of the manuscript; and all authors: critically revised the manuscript and contributed intellectually to its development, provided final approval of the submitted manuscript, had full access to all of the data in the study, took responsibility for the integrity of the data and the accuracy of the data in the analysis, and affirmed that the article is an honest, accurate, and transparent account of the study reported and that no important aspects of the study were omitted. Douglas Laboratories was not involved in design-
ing the study; conducting the study; the collection, management, analysis, or interpretation of the data; or preparation and review of the manuscript before submission. AT received funding in the past 5 y as principal investigator from the Canadian Institutes for Health Research, the Natural Sciences and Engineering Research Council of Canada, the Fonds de recherche du Québec–Santé, and the Fondation de l’Institut universitaire de cardiologie et de pneumologie de Québec as well as investigator-initiated funding from Johnson & Johnson Medical Companies for studies unrelated to the current report. BL is chair of Nutrition at Université Laval, which is supported by private endowments from Pfizer, La Banque Royale du Canada, and Provigo-Loblaws. BL has received funding in the past 5 y from the Canadian Institutes for Health Research, the Natural Sciences and Engineering Research Council of Canada, Agriculture and Agri-
Food Canada (Growing Forward program supported by the Dairy Farmers of Canada, the Canola Council of Canada, the Flax Council of Canada, and Dow Agrosciences), the Dairy Research Institute, Dairy Australia, the Danone Institute, Merck Frosst, Pfizer, and Atrium Innovations, for which Douglas Laboratories manufacture and market ω-3 supplements. BL serves as the chair of the peer-review Expert Scientific Advisory Council of the Dairy Farmers of Canada, an advisory board member of the Canadian Nutrition Society and the Conseil pour les initiatives de progrès en alimentation, and has served as advisory expert for the Saturated Fat panel of the Heart and Stroke Foundation of Canada. BL has received honoraria from the International Chair on Cardio-
metabolic Risk, the Dairy Farmers of Canada, and the World Dairy Platform as an invited speaker in various conferences. PC has received funding in the past 5 y from the Canadian Institutes for Health Research, Agriculture and Agri-
Food Canada (Growing Forward program supported by the Dairy Farmers of Canada, the Canola Council of Canada, the Flax Council of Canada, and Dow Agrosciences), the Dairy Research Institute, Dairy Australia, the Danone Institute, Merck Frosst, Pfizer, and Atrium Innovations. None of the remaining authors had any disclosures to report.
REFERENCES


Effect of DHA-rich fish oil on PPARγ target genes related to lipid metabolism in type 2 diabetes: A randomized, double-blind, placebo-controlled clinical trial.

J Clin Lipidol. 2015
Effect of DHA-rich fish oil on PPAR\(_\gamma\) target genes related to lipid metabolism in type 2 diabetes: A randomized, double-blind, placebo-controlled clinical trial

Anahita Mansoori, Gity Sotoudeh, PhD, Mahmoud Djalali, PhD, Mohammad-Reza Eshraghian, PhD, Mohammad Keramatipour, PhD, Ensieh Nasli-Esfahani, MD, Farzad Shidfar, PhD, Ehsan Alvandi, MSc, Omid Toupchian, Fariba Koohdani, PhD*

Cellular and Molecular Nutrition Department, School of Nutritional Sciences and Dietetics, Tehran University of Medical Sciences, Tehran, Iran (Drs Mansoori, Djalali, Alvandi, Toupchian, and Koohdani); Community Nutrition Department, School of Nutritional Sciences and Dietetics, Tehran University of Medical Sciences, Tehran, Iran (Dr Sotoudeh); Epidemiology and Biostatistics Department, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran (Dr Eshraghian); Medical Genetics Department, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran (Dr Keramatipour); Diabetes Research Center, Endocrinology and Metabolism Clinical Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran (Drs Nasli-Esfahani and Koohdani); and Nutrition Department, Iran University of Medical Sciences, Tehran, Iran (Dr Shidfar)

**KEYWORDS:**
n-3; Gene expression; Peroxisome proliferator–activated receptor \(\gamma\); Lipid metabolism

**BACKGROUND:** The beneficial effects of omega-3 polyunsaturated fatty acids on lipid levels are well documented. However, the related molecular mechanisms are widely unknown. Omega-3 polyunsaturated fatty acids are natural ligand for peroxisome proliferator–activated receptor \(\gamma\) (PPAR\(\gamma\)).

**OBJECTIVE:** The aim of this study was to evaluate the effect of docosahexaenoic acid (DHA)-rich fish oil supplementation on modulation of some PPAR\(\gamma\)-responsive genes related to lipid metabolism.

**METHODS:** Patients with type 2 diabetes were randomly assigned to consume either DHA-rich fish oil (containing 2400 mg/d fish oil; DHA: 1450 mg and eicosapentaenoic acid: 400 mg) or placebo for 8 weeks. Lipid profile and glycemic control parameters as well as the gene expression of PPAR\(\gamma\), liver x receptor-a, ATP-binding cassette A1, and CD36 in peripheral blood mononuclear cells were measured at baseline and after 8 weeks.

**RESULTS:** DHA-rich fish oil supplementation resulted in decreased triglycerides (TG) level compared with placebo group, independently of the baseline value of TG (all patients (\(P=0.003\)), hypertriglyceridemic subjects (\(P=0.01\)), and normotriglyceridemic subjects (\(P=0.02\))). Moreover, a higher reduction in TG level was observed in hypertriglyceridemic subjects, comparing to normotriglyceridemic subjects with DHA-rich fish oil supplementation (\(P=0.01\)). Other lipid parameters as well as the expression of PPAR\(\gamma\), liver x receptor-a, ATP-binding cassette A1, and CD36 were not affected by

G.S. and F.K. contributed equally to this work.
* Corresponding author. School of Nutritional Sciences and Dietetics, Tehran University of Medical Sciences, P.O. Box: 14155-6117, Tehran, Iran.

E-mail address: fkoohdan@sina.tums.ac.ir
Submitted July 11, 2015. Accepted for publication August 22, 2015.

1933-2874/© 2015 National Lipid Association. All rights reserved.
http://dx.doi.org/10.1016/j.jacl.2015.08.007
DHA-rich fish oil supplementation. Only in hypertriglyceridemic subjects, DHA-rich fish oil supplementation upregulated CD36 expression, compared with the placebo group ($P = .01$).

**CONCLUSIONS:** DHA-rich fish oil supplementation for 8 weeks increased CD36 expression in hypertriglyceridemic subjects, which might result to higher reduction in TG level, comparing with normotriglyceridemic subjects. However, this finding should be investigated in further studies.

© 2015 National Lipid Association. All rights reserved.

---

**Introduction**

Peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear transcription factor, which regulates genes involved in lipid metabolism, fat storage, adipocyte function, and insulin action, including adipocyte fatty acid-binding protein, phosphoenolpyruvate carboxykinase, lipoprotein lipase (LPL), the uncoupling protein (UCP1), liver x receptor-a (LXRA), and fatty acid translocase/CD36 (CD36). Polyunsaturated fatty acids (PUFAs) are considered as natural ligands of PPARγ. It has been suggested that docosahexaenoic acid (DHA) might be a more potent activator of PPARγ than eicosapentaenoic acid (EPA). Studies in cell cultures and experimental models as well as human studies suggest that n-3 PUFAs improve lipid metabolism and anti-inflammatory/antioxidant capacity through the regulation of genes related to PPARγ signaling. However, the influence of n-3 PUFAs on a number of PPARγ-related genes involved in lipid metabolism remains to be discovered.

Patients with type 2 diabetes mellitus (T2DM) are frequently affected by coronary heart diseases. Hypertriglyceridemia is the most common lipoprotein alteration in diabetic patients, often accompanied by a decrease in high-density lipoprotein cholesterol (HDL-c). Therefore, impaired reverse cholesterol transport (RCT) has been implicated. In RCT process, excess cholesterol is removed from peripheral cells, and returned to the liver for clearance into the bile and ultimately the feces. The membrane-associated ATP-binding cassette A1 (ABCA1) has pivotal role in RCT. ABCA1 protein mediates cholesterol efflux from cells to apolipoprotein A-1, and promotes the formation of high-density lipoprotein (HDL) particles. Functional mutation in the ABCA1 gene lead to very low level of circulating HDL in both Tangier disease and familial HDL deficiency. ABCA1 expression in peripheral blood leukocyte was correlated negatively with circulating glycated hemoglobin and glucose level, and also reduced in T2DM compared with control group. ABCA1 expression in several tissues is upregulated by LXRA. Human LXRA gene promoter contains functional peroxisome proliferator response element (PPRE), and LXRA transcription is stimulated by PPARγ agonist. Some anti-lipid drugs, PPARγ agonist, and curcumin promote lipid metabolism via PPARγ-LXRA-ABCA1 pathway.

CD36 is a lipid scavenger receptor with the broad cell type expression. The ability of this receptor to recognize and internalize modified forms of low-density lipoprotein (LDL), including oxLDL, is well documented. Upregulation of this receptor leads to increased uptake of oxLDL. The role of CD36 in the intestinal lipid absorption, chylomicron formation and secretion is also reported in many studies. In the proximal intestine, lipid binding to CD36 leads to chylomicron secretion. Moreover, CD36 is involved in the downstream signaling, which promotes the production of large triglyceride (TG)-rich lipoproteins that are rapidly cleared in the blood. Thus, CD36 may be targeted for reducing the postprandial hypertriglyceridemia. In addition, CD36 expression is upregulated by PPARγ.

A comprehensive meta-analysis reported that administration of 4 g/d of n-3 PUFAs leads to decreased TG level by 25–30%, while producing an increase in low-density lipoprotein cholesterol (LDL-c) and HDL-c levels of 10% and 1% to 3%, respectively. However, the gene expression modulations by which n-3 PUFAs affect lipid metabolism are not well documented. Therefore, the aim of the present study was to investigate whether DHA-rich fish oil supplementation mediates the modulation of some PPARγ-responsive genes related to lipid metabolism.

**Materials and methods**

**Participants and study design**

This parallel randomized, double-blind, placebo-controlled clinical trial was conducted on 72 T2DM patients in Tehran, Iran, during January to December 2014. The inclusion criteria were: age 30 to 70 years, and a fasting blood glucose concentration ≥126 mg/dL. The exclusion criteria included the following: (1) unwillingness or inability to participate; (2) insulin or thiazolidinediones therapy; (3) pregnancy, lactation, or hormone replacement therapy; (4) clinical disease that could be related to n-3 metabolism (eg, renal, hepatic, thyroid and coagulation disorders, and malignancies); (5) the history of allergic reaction to fish or fish oil; (6) intake of n-3 supplement within last 3 months; (7) intake of vitamin D, A, and B6 supplement that could influence PPAR activity; (8) any change in the type or dosage of medications during the study.
All 72 participants were stratified based on gender (male or female) and age (<50 or ≥50 years), using random number table to allocate into DHA-rich fish oil supplement or placebo group. The odorless DHA-rich fish oil softgels (containing 600 mg fish oil; DHA: 362.5 mg and EPA: 100 mg; vitamin E [mixed tocopherols]: 1 international unit [IU] in each softgel) known as DHA Ultimate product were provided by Pure Encapsulation Co (Boston). Placebo softgels containing paraffin oil with the same shape, size, and color as the supplements were provided by Zahravi Pharm Co (Tabriz, Iran). The participants were instructed to consume 4 soft gels per day (containing 2400 fish oil; DHA: 1450 mg and EPA: 400 mg or 2400 mg paraffin oil), 2 soft gels after both breakfast and dinner for 8 weeks.

On the first visit, the study protocol was completely described to the participants, and the written informed consent form was obtained from them. After that, a general questionnaire including demographic data, diseases history, and medication use was completed.

On the first day of the intervention, patients received 1 bottle of supplement (containing 120 softgels), which they needed for about 4 weeks of consumption. They were asked to bring the first bottle at the end of 4 weeks and receive the second one. The patients obligated to note the number of taken softgels for each day during 8 weeks. Compliance was assessed by checking the consumption notes, counting the eaten softgels, and making biweekly phone call. The study was conducted according to the guidelines of the Declaration of Helsinki. The ethics committee of Tehran University of Medical Sciences approved the study, and the trial was registered at the Iranian registry of clinical trial Web site (www.irct.ir) as IRCT2013071914013N1.

Assessment of dietary intake, physical activity, and anthropometric parameters

To verify whether the participants maintained dietary intake at a constant level during the intervention, dietary intake of participants was assessed by means of 3-day food diaries (including a weekend day) completed in the first and the last weeks of the intervention period. All participants were given written and verbal instruction with some examples by a dietitian to complete the food records. Dietary energy values and nutrient contents from 3-day food diaries were calculated using Nutritionist IV software (First Databank, San Bruno, CA), modified for Iranian foods. Participants were instructed to maintain a constant level of physical activity restricted to walking, and they were advised not to engage in any form of strenuous physical activity throughout the study, and follow-up of lifestyle was done by biweekly phone call. The classified physical activity questionnaire according to metabolic equivalent tasks was used to assess the amounts of daily physical activity at the baseline and last day of study. Anthropometric measurements were performed by the same person, after overnight fasting with a light cloth and without shoes. Body weight was measured to the nearest of 0.1 kg by the use of digital scale (Seca). Height was measured in relaxed position and freely hanged arms using a stadiometer to the nearest of 0.1 cm (Seca). Body mass index was calculated as body weight (kg) divided by the square of the height (m).

Laboratory assessment

After fasting for 12 hours, the participants’ blood samples were collected in tubes either with or without an anticoagulant (ethylenediaminetetraacetate). TG, total cholesterol (TC), LDL-c, HDL-c, and fasting blood sugar were measured on Sera from clot samples by enzymatic colorimetric assays, using commercially available kits (Pars Azmoon, Iran) and an auto-analyzer (BioLis 24i Premium, Japan). Glycated hemoglobin was measured by using high performance liquid chromatography method (Tosoh, Japan). Serum insulin was measured by using an ELISA kit (Monobind, CA). Homeostasis model assessment—estimation of insulin resistance was calculated based on suggested formula.33

Isolation of RNA and real-time polymerase chain reaction assays

Peripheral blood mononuclear cells were isolated from anticoagulant-treated blood by Ficoll standard density gradient centrifugation. RNA was extracted and purified using Hybrid-R Blood RNA Kit (GeneAll, Seoul, Korea) according to the company’s protocol. The extracted RNA was checked for quality and purity by spectrophotometer (NanoDrop, Thermo Scientific). RNAs from all the subjects were reverse transcribed with the use of a cDNA synthesis kit (Thermo Scientific). Standard real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was carried out in StepOne System (Applied Biosystems, Foster City) with SYBR Green method (Takara Bio Inc, Japan). The primer sequences used for the real-time PCR are summarized in Table 1. GAPDH was used as housekeeping gene. LinRegPCR software was used to determine

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Sequences (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARγ</td>
<td>F: GAATTAGATGACAGCCGACCTG&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>LXRα</td>
<td>R: GCTTCACCACCTTCTTGATGTC</td>
</tr>
<tr>
<td>ABCA1</td>
<td>F: GCCACGACATACCTGCCACAG&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD36</td>
<td>R: TGGATCTGAGGAAAGACTGCTG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: GAGACTGTGTTGTCCTACAGG&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 1  Nucleotide sequences of primers for quantitative real-time polymerase chain reaction
PCR efficiency. The fold change of gene expression was computed using Pfaffl method.

**Statistical analyses**

The normality of data distribution was tested by using Kolmogorov-Smirnov test. The square root transformation was used in the presence of skewness. Student’s independent t-test was used to detect differences in participants’ characteristics, dietary intakes, and gene expression. One-way repeated measures analysis of variance was performed to detect differences in dietary intakes. The effect of treatment on lipid profile was analyzed by analysis of covariance adjusted for baseline values. We performed sub-analyses in normotriglyceridemic and hypertriglyceridemic subjects (hypertriglyceridemia defined as TG level > 150 mg/dL at the baseline of study) to evaluate whether the gene expression response to DHA-rich fish oil depends on the baseline of TG levels. Data were analyzed using SPSS (version 18; Chicago, IL), and P value < .05 was considered significant.

**Results**

Throughout the study, 3 participants in the placebo group were excluded for the following reasons: traveling to abroad (n = 1), detection of colon cancer (n = 1), and poor compliance (n = 1). One participant in DHA-rich fish oil group was also excluded due to gastrointestinal distress. Eventually, 68 participants completed the study. The baseline characteristics of the study population are summarized in Table 2. There were no statistical differences in baseline characteristics (Table 2). Furthermore, no significant difference was seen between comparable groups in terms of dietary intakes of energy, carbohydrate, protein, or fat in baseline and throughout the study (Table 3). DHA-rich fish oil supplementation resulted in decreased TG level compared with placebo group, independent of the baseline value of TG (all patients [P = .003], hypertriglyceridemic subjects [P = .01], and normotriglyceridemic subjects [P = .02]; Table 4). Moreover, a higher reduction in TG level was observed in hypertriglyceridemic subjects, compared with normotriglyceridemic subjects with DHA-rich fish oil supplementation (P = .01). TC, LDL-c, and HDL-c were not significantly affected by DHA-rich fish oil. The expression of the genes in the peripheral blood mononuclear of DHA-rich fish oil group compared with placebo group is shown in Figure 1. In data analysis of all patients, the PPARγ, LXRA, ABCA1, and CD36 showed a similar level of expression in both groups (P > .05) indicating that DHA-rich fish oil supplementation had no effect on the gene expression of PPARγ, LXRA, ABCA1, and CD36 (Fig. 1A). Moreover, the same results were observed in normotriglyceridemic and hypertriglyceridemic subjects (Fig. 1B and C), except for hypertriglyceridemic subjects, in which DHA-rich fish oil supplementation upregulated CD36 expression, compared with the placebo group (P = .01; Fig. 1B).

**Discussion**

The results of the present study confirmed that supplementation with a moderate dose of DHA-rich fish oil, independent of baseline value of TG, could reduce TG level in patients with T2DM. Moreover, CD36 expression was upregulated in hypertriglyceridemic subjects after 8 weeks of supplementation with DHA-rich fish oil. No other significant effects of this supplementation on TC, LDL-c, and HDL-c, and also PPARγ, LXRA, and ABCA1 expression were observed.

Supplementation of all subjects as well as normotriglyceridemic and hypertriglyceridemic subjects with DHA-rich

<table>
<thead>
<tr>
<th>Table 2 Clinical characteristics of patients with type 2 diabetes who received either DHA-rich fish oil supplements or placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DHA-rich fish oil (n = 35)</strong></td>
</tr>
<tr>
<td><strong>Age (y)</strong></td>
</tr>
<tr>
<td>55.8 ± 7.6</td>
</tr>
<tr>
<td><strong>Weight (kg)</strong></td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
</tr>
<tr>
<td><strong>HbA1c (%)</strong></td>
</tr>
<tr>
<td><strong>Physical activity (MET-h/d)</strong></td>
</tr>
<tr>
<td><strong>FBS (mg/dL)</strong></td>
</tr>
<tr>
<td><strong>Fasting serum insulin (mU/L)</strong></td>
</tr>
<tr>
<td><strong>HOMA-IR</strong></td>
</tr>
<tr>
<td><strong>Triglycerides (mg/dL)</strong></td>
</tr>
<tr>
<td><strong>Total cholesterol (mg/dL)</strong></td>
</tr>
<tr>
<td><strong>LDL-c (mg/dL)</strong></td>
</tr>
<tr>
<td><strong>HDL-c (mg/dL)</strong></td>
</tr>
</tbody>
</table>

BMI, body mass index; DHA, docosahexaenoic acid; FBS, fasting blood sugar; HbA1c, glycated hemoglobin; HDL-c, HDL-cholesterol; HOMA-IR, homeostatic model assessment—estimation of insulin resistance; LDL-c, LDL-cholesterol; MET, metabolic equivalent task.

Data are expressed as means ± standard deviation.

*Comparison of baseline characteristics between DHA-rich fish oil and placebo groups derived from unpaired t-test.
Dietary intakes of patients with type 2 diabetes who received either DHA-rich fish oil supplements or placebo throughout the study

<table>
<thead>
<tr>
<th></th>
<th>DHA-rich fish oil (n = 35)</th>
<th>Placebo (n = 33)</th>
<th>(P^*)</th>
<th>(P^i)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal/d)</td>
<td>1606 ± 595</td>
<td>1538 ± 613</td>
<td>.91</td>
<td>.56</td>
</tr>
<tr>
<td>Carbohydrate (% of energy)</td>
<td>59.3 ± 12.7</td>
<td>62.1 ± 10.9</td>
<td>.32</td>
<td>.20</td>
</tr>
<tr>
<td>Protein (% of energy)</td>
<td>15.5 ± 4.7</td>
<td>16.0 ± 5.3</td>
<td>.80</td>
<td>.91</td>
</tr>
<tr>
<td>Total fat (% of energy)</td>
<td>25.7 ± 14.6</td>
<td>23.7 ± 10.7</td>
<td>.55</td>
<td>.45</td>
</tr>
<tr>
<td>SFA (% of energy)</td>
<td>6.9 ± 4.8</td>
<td>6.3 ± 4.5</td>
<td>.34</td>
<td>.29</td>
</tr>
<tr>
<td>MUFA (% of energy)</td>
<td>6.2 ± 3.4</td>
<td>6.0 ± 3.2</td>
<td>.52</td>
<td>.65</td>
</tr>
<tr>
<td>PUFA (% of energy)</td>
<td>7.9 ± 9.8</td>
<td>6.3 ± 6.8</td>
<td>.34</td>
<td>.31</td>
</tr>
<tr>
<td>Cholesterol (mg/d)</td>
<td>214 ± 261</td>
<td>217.8 ± 253</td>
<td>.23</td>
<td>.22</td>
</tr>
<tr>
<td>Dietary fiber (g/d)</td>
<td>9.6 ± 4.5</td>
<td>9.4 ± 4.6</td>
<td>.12</td>
<td>.47</td>
</tr>
</tbody>
</table>

DHA, docosahexaenoic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

*Comparison of between-groups difference at the baseline derived from unpaired t-test.
†Comparison of within-groups difference derived from 1-way repeated measures analysis of variance.

Table 4 Changes of serum lipid concentration in patients with type 2 diabetes throughout the study

<table>
<thead>
<tr>
<th></th>
<th>All patients</th>
<th>Hypertriglyceridemic subjects</th>
<th>Normotriglyceridemic subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DHA-rich fish oil (n = 35)</td>
<td>Placebo (n = 33)</td>
<td>(P^*)</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>(-49.3 ± 87.5)</td>
<td>15.9 ± 63.7</td>
<td>.003</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>(-1.6 ± 20.7)</td>
<td>(-5.9 ± 26.9)</td>
<td>.28</td>
</tr>
<tr>
<td>LDL-c (mg/dL)</td>
<td>3.1 ± 20.0</td>
<td>1.4 ± 16.8</td>
<td>.48</td>
</tr>
<tr>
<td>HDL-c (mg/dL)</td>
<td>(-2.6 ± 9.3)</td>
<td>(-4.6 ± 6.8)</td>
<td>.30</td>
</tr>
</tbody>
</table>

DHA, docosahexaenoic acid; HDL-c, HDL-cholesterol; LDL-c, LDL-cholesterol.

Data are expressed as means ± standard deviation.

*\(P\) values were derived from analysis of covariance with baseline values used as a covariate in all analyses.
involved in the intestinal lipid absorption, chylomicron formation and secretion, and may be targeted for reducing the postprandial hypertriglyceridemia. In addition, CD36 is one of PPARγ target genes. Therefore, reduction of TG may contribute to n-3 PUFA impact (as PPARγ ligand) on PPARγ activation, which leads to the elevated CD36 expression.

N-3 PUFAs, as ligands of PPARγ, upregulate CD36 expression. However, this is due to PPARγ activation, rather than the upregulation of PPARγ expression. In line with previous findings, in the present study, CD36 up-regulation was not accompanied with increased level of PPARγ expression. Furthermore, although we did not assay PPARγ activation per se, CD36 upregulation by DHA-rich fish oil might indicate increased PPARγ activation. However, additional research is required to confirm this result and fully understand the associated mechanisms, and we suggest measuring PPARγ activity in the future studies. Moreover, inflammatory status may affect CD36 expression; thus, we also suggest that pro-inflammatory cytokines can be measured in order to more accurately interpret the data.

In conclusion, DHA-rich fish oil supplementation for 8 weeks reduced TG level in all subjects as well as normotriglyceridemic and hypertriglyceridemic subjects. Furthermore, DHA-rich fish oil supplementation increased CD36 gene expression in hypertriglyceridemic subjects, which might result in a higher reduction in TG level, compared with normotriglyceridemic subjects. However, this finding should be investigated in further studies.

Acknowledgment

This study was supported by Tehran University of Medical Sciences grant 20454-16-01-92, Endocrinology and Metabolism Research Institute grant 1524, and The Iran National Science Foundation (INSF) grant 92031712. The authors would like to acknowledge with gratitude the gift of DHA supplement from Pure Encapsulations, Inc. (Boston, USA). Moreover, the authors would like to express their gratitude to the subjects for their willingness to participate.

References


46. Vallv ...
Mansoori et al  DHA supplementation and gene expression  777


Effect of high dose fish oil supplementation on cerebral blood flow and cognitive performance in patients with mild cognitive impairment: a proof of concept study.
EFFECT OF HIGH DOSE FISH OIL SUPPLEMENTATION ON CEREBRAL BLOOD FLOW AND COGNITIVE PERFORMANCE IN PATIENTS WITH MILD COGNITIVE IMPAIRMENT: A PROOF OF CONCEPT STUDY

O. van de Rest¹, J.A. Claassen², R.P.C. Kessels²,3,4, J.M. Geleijnse¹, M.G.M. Olde Rikkert², L.C.P.G.M. de Groot¹

Abstract: Objective: The current study aimed to examine the effect of n-3 polyunsaturated fatty acids (PUFAs) on cerebral blood flow and age-related loss of cognitive functioning in subjects diagnosed with mild cognitive impairment (MCI). Design, setting and participants: A total of 20 patients with single or multiple domain MCI took part in this randomized, double-blind, placebo-controlled trial, wherein we investigated the effects of four weeks of daily supplementation with either a high pharmacological dose of 3 g eicosapentaenoic acid (EPA) plus docosahexaenoic acid (DHA) (n=10) or placebo (n=10) on cerebral blood flow and cognitive performance. Measurements: Cerebral blood flow and cortical oxygenation were measured by Transcranial Doppler and Near Infrared Spectroscopy together with blood pressure measurements using Finapres. Cognitive function was assessed by sensitive neuropsychological tests measuring reaction times, episodic memory and attention. Results: Daily supplementation with EPA+DHA for 4 weeks had no effect on cerebral hemodynamics or blood pressure. All subjects improved on most of the neuropsychological tests, but there was no difference between the fish oil and the placebo groups. Conclusions: In this randomized controlled trial in MCI patients, daily supplementation with EPA+DHA for four weeks had no effect on cerebral hemodynamics, blood pressure, or cognitive performance.

Key words: EPA, DHA, cerebral blood flow, cognitive functioning, MCI patients.
DHA is the predominant n-3 fatty acid in the brain and an integral component of neural membrane phospholipids (9). Both EPA and DHA may reduce oxidative stress, are anti-inflammatory and have been linked with neurotransmission, membrane fluidity, ion channel and enzyme regulation and gene expression (10). N-3 PUFA have also been linked to vascular risk factors, which also contribute to the development and progression of AD (11). The effect of cerebrovascular disease might also be caused by impairments in cerebral hemodynamics, such as a decline in cerebral blood flow (CBF) with advancing age (12). Several studies have described the influence of n-3 PUFA on cerebral circulation and this includes effects on reductions in platelet agonist thromboxane A2 synthesis (13), blood pressure (14), epinephrine and norepinephrine concentrations (15), and blood viscosity (16). Two studies observed that EPA might increase the oxygenation level (17) and that DHA supplementation increased oxygenated hemoglobin (Hb) and total levels of Hb, indicative of increased CBF in healthy adults (18).

In the present study, we assessed the effect of supplementation with a high dose challenge of EPA+DHA on cerebral blood flow, cortical oxygenation, and cognitive performance in subjects with MCI.

Methods

Participants

Patients with amnestic MCI were recruited by the Radboud Alzheimer Centre Nijmegen and hospital Gelderse Vallei at Ede, the Netherlands. Men and women diagnosed as having amnestic (single or multiple) MCI were included. The diagnosis of MCI was based on a multidisciplinary approach, including extensive cognitive testing, neuroradiological findings and medical examination. Cognitive impairments had to be present in one or more cognitive domains (cut-off of 1 standard deviation below age and education adjusted normative mean, including episodic memory (single or multiple-domain amnestic MCI, according to Busse A et al (19)). Furthermore, patients were excluded if 1] no principal caregiver willing to assist for a successful participation was present; 2] they used fish oil supplements; 3] they consumed fish >2 times/week; 4] they used dementia (Alzheimer) medication, Acenocoumarol or other anti-thrombotic drugs; 5] they had a serious liver disease; or 6] they consumed >4 glasses of alcohol per day. After screening, eligible subjects started with a run-in period of one week to get used to swallowing six capsules/day. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human patients were approved by the Medical Ethical Committee region Arnhem-Nijmegen. Written informed consent was obtained from all patients.

Sample size calculation was based on the Digit Span test. A difference in score of 2 points on the Digit Span test was considered clinically relevant. The mean and SD for non-cognitively impaired elderly are 8.5 and 2.0 respectively (20). With these assumptions we calculated that a minimum sample size of 17 participants per group would be required to detect an effect with a power of 80% at a significance level of 0.05. Taking into account an anticipated dropout rate of 15%, 20 participants per treatment group would be needed. However, after two years of extensive screening only 20 eligible participants could be recruited. According to a post-hoc power calculation this provided adequate power to detect an effect size of d= 1.33 with all cases included in the analyses.

Intervention

For the intervention period of four weeks, participants were randomly allocated to either treatment with capsules with a high dose challenge of 3 g EPA/DHA or placebo capsules. Randomization was performed by an independent person and taking into account stratification by gender and APOE4 status, i.e. carriers and non-carriers of the APOE4 allele. All subjects and researchers were blinded towards the type of treatment assigned to subjects until the end of the study, after blind review of the data had been completed. The oils were administered in six white-colored soft gelatin capsules daily, each containing 900 mg fish oil (EPA:DHA =3:2) and 29 mg of a natural tocopherol mix as antioxidant, or a placebo oil (sunflower oil) (Orthica, Almere, the Netherlands).

Compliance was checked by counting returned capsules and by inspecting the diaries that subjects kept throughout the study to report the intake of the capsules. A participant was considered to be compliant if >80% of the capsules were taken. An objective measure of compliance was obtained by measuring the n-3 fatty acid composition in the serum cholesteryl esters, which directly reflects intake over the past weeks (21).

Cerebral blood flow (CBF), cortical oxygenation, and blood pressure

We used Transcranial Doppler (TCD) to assess cerebral blood flow at baseline and after four weeks of intervention. TCD applies ultrasound with high temporal resolution to measure changes in cerebral blood flow velocity (CBFV) in the middle cerebral artery accessed through the temporal bone ("the temporal window"). Under the assumption that the vessel diameter is constant, changes in CBFV (Cerebral blood flow velocity) represent changes in CBF. In addition, Near-Infrared Spectroscopy (NIRS) (Oxymon, Artinis Medical Systems, The Netherlands) was used to measure cerebral cortical...
oxygenation over the frontal cortices. NIRS uses light at near infrared wavelengths to monitor changes in oxygenated and deoxygenated cerebral Hb concentrations. For this purpose, optodes were placed on the skin of the skull overlying the cortical region of interest. Finally, we assessed arterial blood pressure using Finapres (Finapres Medical Systems, Amsterdam).

We used these measures to look at changes between baseline and after four weeks of intervention in blood pressure, CBF, and cortical oxygenation. In addition to these steady-state hemodynamics, we used spectral analysis of the beat-to-beat time series of blood pressure, CBFV and oxygenated Hb to investigate possible changes in spontaneous oscillations (very low frequency, low frequency and high frequency) in these parameters. Oscillations in these frequency bands reflect cardiovascular autonomic control (baroreflex function, parasympathetic and sympathetic action) (22, 23). The transfer of these oscillations in blood pressure into oscillations in cerebral blood flow reflects cerebral autoregulation that in turn is influenced by cerebrovascular smooth muscle function, endothelial function, and vessel stiffness. Transfer function analysis was thus used to investigate cerebral autoregulation by comparing changes in cerebral blood flow oscillations relative to changes in blood pressure oscillations (22, 24-26). In addition, transfer function analysis was used to calculate the phase difference between blood pressure oscillations and oscillations in oxygenated Hb. This phase shift is an important marker for effective autoregulation, and is sensitive to changes in cerebrovascular properties (26, 27).

Neuropsychological tests

Cognitive performance was assessed at baseline and after four weeks of intervention by means of several sensitive neuropsychological tests focusing on memory, reaction times and attention/working memory. As part of the screening the Mini-Mental State Examination (28) and Clock Drawing test (29) were performed.

The forward test of Wechsler’s Digit Span task was included as a measure of attention and the backward test as an index of working memory (30). Two subtests of the computerized Test of Attentional Performance (TAP) were administered: Alertness and Flexibility (31). The Alertness test measures simple and cued reaction time and the Flexibility test measures selective attention. The Paired Associate Learning (PAL) subtest of the Wechsler Memory Scale-Revised (WMS-R) was included to assess episodic (associative) memory (32). The total battery of tests required on average 30 minutes.

Blood

During the screening a blood sample was collected into a 10 ml EDTA-vacutainer and stored at -20°C for APOE genotype determination by the polymerase chain reaction-based restriction fragment length polymorphism method and restriction enzyme digestion with Hha 1 (33) to determine APOE4 status. At baseline and after four weeks of intervention 10 ml blood was collected to determine omega-3 fatty acid composition in cholesteryl esters. Fatty acid composition in cholesteryl esters was analyzed by gas chromatography as described previously (34) at the laboratory of the Division of Human Nutrition, Wageningen University.

Other measurements

Weight was measured to the nearest 0.5 kg with the person dressed in light clothing and without shoes. Height was measured to the nearest 0.1 cm with the person in standing position and wearing no shoes. Information on educational level, smoking behavior, alcohol and fish consumption, medical history and current use of medication was obtained by a structured questionnaire during a personal interview. During the study participants had to report any adverse events in a diary.

Statistical analyses

Differences in baseline values (for all parameters) between the two treatment groups were analyzed with an independent t-test (continuous variables) or chi-square analysis (categorical variables). Changes from baseline to the end of the study within each treatment group, for hemodynamic and cognitive tests, were analyzed using paired samples t-tests. Changes between the fish oil and the placebo group were compared with the independent samples t-test. Alpha was set at 0.05 (two-tailed testing). Statistical analyses were performed using PASW Statistics 18.0.3.

Results

Eligible participants were screened between October 2008 and October 2010 and intervention took place between December 2008 and December 2010. Figure 1 shows the participant flow through the study. Of all subjects only one subject (who was in the fish oil group) dropped out, because of not feeling well. Apart from the individual who stopped treatment prematurely, the average adherence to treatments based on counts of returned capsules was 94%. Compliance was confirmed by a change in the proportion of EPA+DHA in plasma cholesteryl esters of 321% in the fish oil group (from 1.66
± 0.61 to 6.98 ± 0.074 to 1.66 ± 0.61 g/100g fatty acids) and -5.6% (from 1.25 ± 0.074 to 1.20 ± 0.074 g/100g fatty acids) in the placebo group. The supplements were well tolerated; adverse events were reported by two participants, one in the fish oil group (feeling warm at night) and one in the placebo group (itch and joint pain). Mean age of the participants was 73.1 ± 8.8 years and 45% were male. The two treatment groups were similar with regard to baseline characteristics as presented in Table 1.

Figure 1
Flow of participants through study

Table 1
Characteristics of 20 subjects with MCI participating in a randomized, placebo-controlled trial, by treatment group*

<table>
<thead>
<tr>
<th></th>
<th>Fish oil (n=10)</th>
<th>Placebo (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>72.6 ± 10.8†</td>
<td>73.6 ± 6.8</td>
</tr>
<tr>
<td>Sex, Male (%)</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>Education Low/ Middle/ High (%)</td>
<td>10/ 50/ 40</td>
<td>20/ 40/ 40</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.9 ± 4.1</td>
<td>26.3 ± 4.2</td>
</tr>
<tr>
<td>Smoking behavior (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>Former smokers</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Alcohol consumers (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol consumption (glasses/ week)</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Fish consumers (%)</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>Fish consumption (times / month)</td>
<td>3 (3-4)</td>
<td>4 (2-6)</td>
</tr>
<tr>
<td>Plasma EPA+DHA (mass/%)</td>
<td>1.7 ± 0.6</td>
<td>1.8 ± 0.7</td>
</tr>
<tr>
<td>APOE ε allele 0/ 1/ 2 (%)</td>
<td>50/ 50/ 0</td>
<td>30/ 70/ 0</td>
</tr>
<tr>
<td>MMSE (Mini-Mental State)</td>
<td>26.0 (24.8-27.5)</td>
<td>26.5 (24.8-27.0)</td>
</tr>
<tr>
<td>Examination</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>24-30</td>
<td>19-30</td>
</tr>
<tr>
<td>Clock drawing test (%) abnormal</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, Body Mass Index; MMSE, Mini-Mental State Examination; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; APOE, apolipoprotein E
* There were no significant differences between the two treatment groups (independent samples t-test for continuous variables and chi-square analysis for categorical variables); † Mean ± SD (all such values); ‡ Mean consumption in consumers only; $ Median (Q1-Q3) (all such values); I Missing value for 1 participant

Cerebral measures

Figures 2-5 show that both groups were comparable at baseline for all steady-state systemic and cerebral measures (blood pressure, heart rate, cerebral blood flow velocity) as well as for the dynamic measures obtained through spectral and transfer function analysis (spectral power of blood pressure oscillations and cerebral blood flow oscillations, and phase shift between blood pressure and cortical oxygenation). The intervention with four weeks of fish oil versus placebo did not result in changes in any steady-state or dynamic measures of blood pressure, cerebral perfusion or cerebrovascular autoregulation.

Figure 2
Effects of fish-oil on heart rate, blood pressure and cerebral perfusion

Legend: mean heart rate, mean arterial blood pressure (MAP) and cerebral blood flow-velocity (CBFV) in subjects with mild cognitive impairment, at baseline (B) and after 4 weeks (4w) of fish oil (shaded bars) or placebo (gray bars). Values are mean, error bars represent standard deviation. There were no significant differences between groups or between baseline and follow-up.

Neuropsychological tests

Baseline scores on the cognitive tests were comparable between the two groups, except for the TAP flexibility tests letters (P=0.03 [95% CI -214 - 943]) and letter-digit alternating (P=0.05 [95% CI -589 - 1622]). After four weeks of intervention participants in both treatment groups improved significantly on the Digit Span forward test and almost all TAP tests, except non-cued Alertness in the fish oil group. Contrarily, on the Digit Span Backward and especially on the Paired Associate Learning test they performed worse, but only in the fish oil group these declines were significant (Table 2). However, compared to the placebo group, participants in the fish oil group did not change significantly, except for TAP Flexibility Letters where the placebo group performed faster after 4 weeks of intervention, P=0.01; -334 [95% CI -805-136] (Independent-samples t-test).
Figure 3
Effects of fish-oil on spontaneous blood pressure oscillations

Figure 4
Effects of fish-oil on spontaneous cerebral blood flow oscillations

Discussion
In this randomized controlled trial in MCI patients, daily supplementation with EPA+DHA for four weeks had no effects on systemic and cerebral hemodynamics. The intervention also had no effect on cognitive performance. All participants improved significantly on most of the neuropsychological tests, but no differences were found between the fish oil and placebo groups.

This study has been designed as a challenge study: to examine rapid changes in response to a high pharmacological treatment dose in a sensitive group of patients. Therefore, the duration of the study is limited, but previous studies showed that near-maximal incorporation of EPA and DHA in serum cholesteryl esters (21), plasma phospholipids and blood mononuclear cells (35) is already reached after four weeks of supplementation. In contrast, we intervened with a rather high dose of fish oil (3 g EPA+DHA daily) in patients with MCI, a transition stage between normal cognitive aging and dementia, characterized specifically by memory impairment beyond that expected for age and education (36). From previous (epidemiological) studies it appears that subjects sensitive to progression, such as MCI subjects, are the preferred group to investigate (37). In comparison to several other trials investigating the effect of fish oil supplementation on cognitive performance our trial is a relatively small study. Our
However, the role of APOE4 status is contradictory and APOE genotype affects responsiveness to EPA+DHA dementia and moreover several studies suggest that on gender and APOE4 status. It is known that carriers of weeks of intervention.


table. The fact that all improved could also be the reason that no additional effect of fish oil compared to placebo was found. Unexpectedly, both groups performed worse on the episodic memory test after 4 weeks of intervention.

In this study stratification was performed based on the Digit Span cognitive tests and at least 17 subjects per group would be required. Unfortunately, despite a long recruitment period of two years, only 10 subjects per group that fulfilled our study criteria and were willing to participate were found. This would, however, still be sufficient to identify an effect size of 1.33. Moreover, the results do not show a trend towards a significant difference. Also, besides studying cognitive performance, we also investigated possible underlying mechanisms by using TCD, NIRS, and Finapres. To study the detailed information that these methods provide requires extensive and time-consuming data analysis and therefore such detailed studies are limited to small numbers of participants, where sufficient power is achieved with sample sizes as small as n= 8 (24-26).

Neuropsychological tests were included to see whether changes in cerebral blood flow would also result in measurable changes on these tests, which are more sensitive than cognitive screens such as the MMSE.

Both groups improved significantly on the neuropsychological tests, which could be due to nonspecific learning effects, although parallel versions were used to take material-specific learning effects into account. The fact that all improved could also be the reason that no additional effect of fish oil compared to placebo was found. Unexpectedly, both groups performed worse on the episodic memory test after 4 weeks of intervention.

In this study stratified randomization was used based on gender and APOE4 status. It is known that carriers of the APOE-ε4 allele have a higher risk of developing dementia and moreover several studies suggest that APOE genotype affects responsiveness to EPA+DHA intake and its subsequent effect on cognitive functioning. However, the role of APOE4 status is contradictory and merits further research as studies show that EPA+DHA intake may either protect against cognitive impairment in non-carriers of the APOE-ε4 allele (38-42) or in carriers (20, 43, 44). In our study there was no significant interaction between APOE4 status and treatment.

To the best of our knowledge there are no other trials studying the effects of fish oil supplementation that included TCD measurements and only two other trials (17, 18) that used NIRS to gain more insight in one of the possible mechanisms explaining the potential association between n-3 fatty acids and cognitive performance. They both observed an increase in oxygenation level after supplementation with either EPA or DHA, which is indicative of an increased CBF. Gaining more insight in vascular disease related mechanisms is of clinical relevance, considering the importance of vascular disease in the etiology of AD, frequent occurrence of cerebrovascular comorbidity in AD, and in the growing interest in the use of fish oil on (vascular) cognitive impairment. However, in this study we did not confirm the earlier observation that supplementation with either EPA or DHA increased CBF. There were no changes in cerebral blood flow-velocity, as measured with TCD in either placebo or intervention group. There was also no indication of more subtle positive effects on the cerebral vasculature, as we observed no changes in spontaneous oscillations in cerebral blood flow, and no effect on cerebral autoregulation as measured with TCD (cerebral blood flow-velocity) and NIRS (oxygenated Hb).

Our method is based on the assumption that an increase in CBF improves cognition. Direct evidence for this assumption is lacking, although the available circumstantial evidence is highly suggestive. In the Rotterdam Study individuals with cognitive decline were found to have lower CBF than the individuals who had stable cognitive function in the previous years (45). The
mechanism for this relationship is likely to be the increasing sensitivity of neurons to ischemia or hypoperfusion with age (46). The evidence for the causal relationship between impairment in CBF, neuronal injury, and cognitive decline has recently been reviewed elsewhere (47, 48). Hooijmans et al studied the effects of dietary lipids (cholesterol and DHA) on Aβ accumulation and brain circulation in mice (49). They observed that the DHA-enriched diet increased relative cerebral blood volume without changing blood flow indicating a larger circulation in the brain probably due to vasodilatation, and decreased the amount of vascular β-amyloid deposition.

We and others have shown earlier that AD is associated with reduced cerebral perfusion and increased cerebrovascular resistance (50-52). More recently, it was shown that MCI patients show changes in cerebral hemodynamics that lie in between normal controls and AD. In addition, we found that AD patients exhibit impairments in cardiovascular autonomic control (baroreflex) and that, again, MCI patients show changes that are between normal controls and AD. Therefore, we argue that cardiovascular and cerebral hemodynamics in our MCI patients were plausible targets that had a theoretical potential for improvement, e.g. in the form of reduction in cerebrovascular resistance, increase in cerebral blood flow, or changes in cardiovascular autonomic control.

In summary, in this randomized controlled trial performed in a small group of MCI patients, we observed no effect of daily supplementation with EPA+DHA for four weeks on cognitive performance, cerebral hemodynamics or blood pressure.

Acknowledgements: We thank all study participants for their time and dedication; William van Aalst for his help in recruiting, screening and motivating the participants; Andre Janse for his help in recruiting patients at Hospital Gelderse Vallei Ede; Arenda Dado-van Beek, Nikita van der Zwaluw, Jantien de Graaf for excellent performance of measurements; and Orthica, Almere, the Netherlands for supplying the fish oil and placebo soft gels. Trial registration: ClinicalTrials.gov, NCT00746005.

Funding: This study was funded by the Netherlands Organization for Health Research and Development (ZonMw, grant number 6100.0004). The Hague, the Netherlands and supplements were supplied by Orthica, Almere, the Netherlands.

References
High potency fish oil supplement improves omega-3 fatty acid status in healthy adults: an open-label study using a web-based, virtual platform.

Nutr J. 2013.
High potency fish oil supplement improves omega-3 fatty acid status in healthy adults: an open-label study using a web-based, virtual platform

Jay K Udani1 and Barry W Ritz2,3*

Abstract

Background: The health benefits of omega-3 fatty acids from fish are well known, and fish oil supplements are used widely in a preventive manner to compensate the low intake in the general population. The aim of this open-label study was to determine if consumption of a high potency fish oil supplement could improve blood levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and impact SF-12 mental and physical health scores in healthy adults.

Methods: A novel virtual clinical research organization was used along with the HS-Omega-3 Index, a measure of EPA and DHA in red blood cell membranes expressed as a percentage of total fatty acids that has been shown to correlate with a reduction in cardiovascular and other risk factors. Briefly, adult subjects (mean age 44 years) were recruited from among U.S. health food store employees and supplemented with 1.1 g/d of omega-3 from fish oil (756 mg EPA, 228 mg DHA, Minami Nutrition® MorEPA® Platinum) for 120 days (n = 157).

Results: Omega-3 status and mental health scores increased with supplementation (p < 0.001), while physical health scores remained unchanged.

Conclusions: The use of a virtual, web-based platform shows considerable potential for engaging in clinical research with normal, healthy subjects. A high potency fish oil supplement may further improve omega-3 status in a healthy population regularly consuming an omega-3 supplement.

Keywords: Omega-3 index, Eicosapentaenoic acid, Docosahexaenoic acid, Fish oil, Open-label

Introduction

Considerable evidence has demonstrated that increasing consumption of omega-3 fatty acids can benefit health, most notably by reducing cardiovascular disease and its associated risk factors [1-5]. Over the past several decades, evidence has emerged indicating that higher intakes of omega-3 are associated with a significant reduction in all-cause mortality [6,7], and a 2006 meta-analysis of randomized controlled trials found that fish oil significantly reduced total mortality [8]. Specifically, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been identified as health promoting. Both EPA and DHA may improve cardiovascular health by several mechanisms. In general, both EPA and DHA compete with arachidonic acid (omega-6) in cyclooxygenase and lipoxygenase synthesis, resulting in net anti-inflammatory effects. Both reduce blood viscosity without a significant effect on platelet or clotting factors, and both have positive effects on blood lipids, most consistently a reduction in triglyceride concentrations [9]. However, some recent analyses have suggested that protection against cardiovascular outcomes may not be as pronounced as indicated by earlier investigations [10,11], raising awareness that additional controlled studies are needed.

* Correspondence: br@atrium-innovations.com
2Atrium Innovations Inc, 4 Hillman Drive, Suite 190, Chadds Ford, PA 19348, USA
3Nutrition Sciences Department, College of Nursing and Health Professions, Drexel University, 245 N. 15th Street, Philadelphia, PA 19102, USA
Full list of author information is available at the end of the article

© 2013 Udani and Ritz; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
Data are also emerging connecting omega-3 fish oils with cognitive function and mental health status [12-16]. Population studies and preliminary clinical data demonstrate that omega-3 fatty acids may help elevate mood in depressed subjects [13,14]. In particular, high-EPA interventions and higher plasma EPA may be associated with improved clinical outcomes [17], although more research is needed in the application of EPA and fish oil in cognitive and mental health.

Despite the clear need for more controlled studies in specific populations and targeting specific clinical outcomes, increasing blood EPA and DHA levels appears to have a generally positive impact on health, and awareness among the general population is high [18]. Because of this, the use of fish oil supplements has increased dramatically over the past decade, reaching sales of $1.145 billion in 2011 and continuing to grow [19]. The American Heart Association recommends an intake of 1 g/d of combined EPA and DHA from food or supplements for secondary prevention [20], and concentrated formulations of EPA and DHA are now available as both dietary supplements and medications.

The aims of the present study were to investigate the utilization of a new web-based program known as a virtual clinical research organization (CRO) and to assess the effects of a high potency fish oil on omega-3 status, as well as mental and physical health scores, in healthy adults who regularly consume an omega-3 supplement. To our knowledge, this represents the first such use of a virtual CRO to assess the impact of a nutritional intervention on a quantifiable biomarker of health in the general population.

Materials and methods
Virtual contract research organization (CRO)
The proprietary, web-based CRO program was developed by Medicus Research (Northridge, CA). This system enabled researchers to enroll subjects through inclusionary and exclusionary dialogue and track and monitor progress by using survey tools and automatic prompts. Researchers virtually followed up with subjects regarding completion of milestone events, adverse event reporting, non-compliance issues, and other study requirements.

Subject recruitment and protocol
The protocol was approved by Western IRB (Olympia, WA) prior to the initiation of any study related activities. Healthy subjects between the ages of 18 and 70 years working at health food stores nationwide were recruited. Subjects (n = 316) were invited via email to participate in the study. Those who accepted the offer were directed to a secure website where they took part in an online informed consent procedure with electronic signature. Participants then completed questions related to standard inclusion and exclusion criteria such as age (18–70 years), agreement to participate, and exclusion based on a history of bleeding disorders, current use of anticoagulant medications, immune disorders, diabetes, planned surgery, or pregnancy, among other criteria. The subjects agreed to not change their current diet or exercise program and to stop using all other dietary supplements, including their present omega 3 fish oil supplement, for a two-week wash-out period before onset of the study and for the duration of the intervention. Subjects were then scheduled to undergo a phone screen with a trained clinical staff member. This phone screening also ensured the subjects understood their rights and responsibilities related to the study prior to their participation.

The study duration was 120 days and featured a total of 5 virtual CRO visits (V1-5). The subjects answered a series of online questionnaires regarding medical history, including an assessment of current supplemental omega-3 intake and web-based SF-12 Health Survey (V1). Subjects then received via mail the first month’s supply of the study product and the Omega-3 Index test kit, which was to be completed on the same day they began taking the study product. The study product provided a total of 1,100 mg omega-3 s with 756 mg EPA, 228 mg DHA, and 300 IU vitamin D3 in a single soft gel capsule (Minami Nutrition® MorEPA® Platinum, provided by Garden of Life, West Palm Beach, FL).

The subjects continued to receive monthly study product via mail, as well as an email reminder from the Virtual CRO for the 30-day (V2), 60-day (V3), 90-day (V4), and 120-day (V5) assessments. The emails directed the subjects to a secure link for completion of the compliance assessment, medical review, SF-12 Health Survey, and adverse event questionnaires. The subjects were also instructed to complete the Omega-3 Index test at V5. Compliance was assessed through the study website where subjects were instructed to enter the number of pills remaining in the study product bottle at the end of each month.

Omega-3 status
Omega-3 status was quantified using the HS-Omega-3 Index, a finger stick blood test that measures the amount of EPA and DHA in red blood cell membranes expressed as the percentage of total fatty acids in the membrane. The test was employed according to manufacturer’s instructions and results were analyzed and reported by the manufacturer (OmegaQuant, Sioux Falls, SD). Briefly, subjects received test kit directly from the laboratory responsible for testing, which includes a large bag, small bag, test request form, collection card, alcohol pad,
adhesive bandage, cotton ball, lancet, desiccant pack, and return envelope. Subjects were instructed to fill out test request form and collection card first and wash hands thoroughly before sterilizing either ring or middle finger with the alcohol pad. Subjects used lancet on sterilized finger and applied drop of blood directly onto collection card, blood was allowed to dry on the card for 15–20 minutes, and card was then folded in half over the spot and placed in the small bag with desiccant. The small bag was placed in the large bag along with the test request form, and then the large bag was placed in return envelope. Subjects were instructed to return the envelope via mail on the same day as collection. The data management team received the results.

SF-12 health survey
The subjective assessment of mental and physical health as composite scores utilized a shortened, validated version of the SF-36 health survey created by Quality Metric and in use since 1994 [21-23]. The scoring system applied was from Positive Aging Resource Center, and the reference range of scores had a cutoff of 50 for Physical Component Summary (PCS) and 42 for Mental Component Summary (MCS).

Statistical analysis
All values are reported as the mean ± the standard error of the mean (SEM) or as a percentage of total subjects, and data were analyzed using SPSS (Chicago, IL). Paired T-tests were used to test significance for the HS-Omega 3 Index (baseline versus day 120), and analysis of variance (1 by 4) was used to determine time effect of the intervention on the SF-12 composite scores.

Results
A total of 316 subjects were recruited for the study, 251 subjects completed a baseline HS-Omega-3 Index assessment, and 157 subjects completed all aspects of the trial through 120 days. Non-completers were lost to follow-up. Adverse events reported were not serious and were determined to not be associated with the study product. The mean age of subjects was 44.3 ± 0.9 years. Table 1 provides information on omega-3 supplement usage at baseline. No other demographic data were obtained.

The HS-Omega-3 Index increased from 6.1% (range of 3.1–11.8%) at baseline to 7.3% (3.7–13.3%) at 120 days (p < 0.001, Figure 1). This represents an approximate 20% increase over baseline. The baseline score of approximately 6% was higher than previously reported baseline scores of approximately 4%, as expected, reflecting regular use of omega-3 fatty acid supplements [24,25]. The mental composite score increased after 60 days and remained elevated through day 120 (p < 0.001, Table 2). There was no change in the physical composite score at any time point during the intervention.

Discussion
To our knowledge, the present study is the first to report a significant increase in blood EPA and DHA levels, as Omega-3 Index, in a group of healthy individuals who were regular consumers of fish oil supplements at baseline. Maximizing the response to omega-3 supplementation may be a prudent clinical target for the reduction of certain risk factors, and Omega-3 Index may be an appropriate biomarker for assessing and monitoring the effectiveness of omega-3 supplementation.

An increase in the Omega-3 Index has been correlated with reductions in cardiovascular risk factors, and low

---

**Table 1 Baseline consumption rates of omega-3 fatty acid supplements**

<table>
<thead>
<tr>
<th></th>
<th>Fish oil capsules</th>
<th>Fish oil liquid</th>
<th>Vegetarian omega-3 capsules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total users¹</td>
<td>66%</td>
<td>21%</td>
<td>19%</td>
</tr>
<tr>
<td>Frequency of use²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/d</td>
<td>51%</td>
<td>72%</td>
<td>36%</td>
</tr>
<tr>
<td>2/d</td>
<td>30%</td>
<td>3%</td>
<td>16%</td>
</tr>
<tr>
<td>3/d</td>
<td>5%</td>
<td>1%</td>
<td>-</td>
</tr>
<tr>
<td>4/d</td>
<td>2%</td>
<td>1%</td>
<td>-</td>
</tr>
<tr>
<td>5+/d</td>
<td>1%</td>
<td>1%</td>
<td>-</td>
</tr>
<tr>
<td>1/wk</td>
<td>11%</td>
<td>21%</td>
<td>47%</td>
</tr>
<tr>
<td>Dosage²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 500 mg</td>
<td>10%</td>
<td>25%</td>
<td>63%</td>
</tr>
<tr>
<td>500–1000 mg</td>
<td>57%</td>
<td>42%</td>
<td>26%</td>
</tr>
<tr>
<td>&gt; 1000 mg</td>
<td>33%</td>
<td>33%</td>
<td>11%</td>
</tr>
</tbody>
</table>

¹Percentage (%) of total study population.  
²Percentage (%) of omega-3 supplement users by column.

---

**Figure 1 Omega-3 Index in healthy subjects at baseline and following fish oil supplementation, *p < 0.001.**
Omeg-3 Index has been reported as a predictor of sudden cardiac death [24-27]. Importantly, it has also been suggested that an Omega-3 Index of greater than 8% was associated with a lower risk for acute coronary syndromes and decreased morbidity and mortality in those with cardiovascular disease [28]. Conversely, an Omega-3 Index less than 4% was associated with a ten-fold increase in risk of cardiac death compared to subjects with an Omega-3 Index greater than 8% [29]. Inflammatory markers were also shown to be inversely related to Omega-3 Index [30], and an Omega-3 Index of 8% or higher was associated with slowed cellular aging as measured by the five-year rate of telomere shortening [31].

In the current study, we demonstrated a significant increase in Omega-3 Index in 120 days of regular supplementation with a high potency fish oil. Assuming a clinical target of >8%, the percentage of subjects meeting this target increased from 5.6% at baseline to 24.8% of subjects following supplementation. Such an increase may be expected to yield clinically relevant outcomes among responders, although this would require further study. It is unknown whether continued supplementation would further raise Omega-3 Index scores beyond what was observed at 120 days or increase the number of subjects achieving a score of >8%, although this seems a reasonable extrapolation of the current data.

The SF-12 health survey provides a quick assessment of an individual’s physical and mental health. The average baseline mental health and physical health scores from this study are similar to those previously reported [32]. In this study, we also detected a small but significant increase in mental health scores based on the SF-12 mental health composite compared to baseline. However, the study design did not include a placebo control, and there are insufficient corroborative data to conclude that omega-3 supplementation and a corresponding increase in Omega-3 Index can improve cognitive function in a healthy population.

The current study had certain limitations in design and execution. First, information was not obtained about the composition of omega-3 fatty acid products being consumed prior to the study, and only a basic analysis of omega-3 intake from food sources was performed (not reported). Subjects were instructed to maintain normal dietary habits, and fish consumption was not restricted. Further, it is possible that the subjects could have self-reported inaccurate information regarding their supplement use; however, over-reporting of supplement use is not likely as their omega-3 status was higher than what has been reported in the typical population. Next, the use of an open-label design creates the potential for bias by both investigator and subject. The virtual CRO is designed to track and monitor the subject compliance with minimal human interaction and influence. While this is a positive, the tradeoff may be in overall compliance. While the absence of a placebo does not allow for the determination of normal changes in blood EPA and DHA levels associated with daily nutritional practices, because the subjects had already been taking a fish oil supplement, the baseline value served as the surrogate placebo or comparator value. Further, it was impractical and perhaps unethical, given the known health benefits of omega-3 consumption, to restrict omega-3 supplementation in a currently supplementing healthy population for a prolonged period of 4 months. Therefore, it was determined that a placebo was not required. Clearly, the lack of a placebo arm does not allow for the assessment of causality in the subjective assessment of mental health scores.

### Table 2 Mental and physical health composite scores in healthy subjects at baseline and following fish oil supplementation

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>60</th>
<th>90</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mental health</td>
<td>49.5 ± 0.5</td>
<td>52.5 ± 0.5*</td>
<td>53.0 ± 0.6*</td>
<td>53.5 ± 0.5*</td>
</tr>
<tr>
<td>Physical health</td>
<td>53.2 ± 0.3</td>
<td>53.1 ± 0.4</td>
<td>53.2 ± 0.4</td>
<td>53.86 ± 0.3</td>
</tr>
</tbody>
</table>

*p < 0.001 compared to baseline.

Overall, the combination of the virtual CRO method, the use of the SF-12, and the self-administered Omega-3 Index finger prick blood test provided an appropriate, effective, and efficient design for assessing the potential impact of a nutritional supplement on biomarkers of health in a non-diseased population. The current study demonstrated significant improvements in blood EPA and DHA status, and a small but significant increase in subjective mental health scores, following the compliant use of a high potency fish oil supplement in a healthy population that had been previously taking a fish oil supplement. Further studies are needed with more rigorous design, extended duration, and the evaluation of clinical endpoints to assess the relevance of high potency fish oil supplementation in the general population. Additional studies should evaluate the effectiveness of the virtual CRO as a research tool in evaluating nutritional interventions in a healthy population, as such studies are grossly lacking.

### Competing interests
The study was sponsored by Garden of Life, West Palm Beach, FL, a subsidiary of Atrium Innovations, Inc. Medicus Research conducted the study independent from the sponsor, such that the sponsor had no influence on the outcome of the study.
Authors’ contributions

Both authors conceived of the design. JKU oversaw the execution of the study and data analysis including statistical analysis. Both authors read and approved the final manuscript.

Acknowledgments

The authors recognize Karolyn Gazella and Kelly Gibson for their assistance in preparing the manuscript.

Author details

1Medicus Research, 28720 Roadside Drive, Suite 310, Agoura, CA 91301, USA.
2Atrium Innovations Inc, 4 Hillman Drive, Suite 190, Chadds Ford, PA 19348, USA.
3Nutrition Sciences Department, College of Nursing and Health Professions, Drewel University, 245 N. 15th Street, Philadelphia, PA 19102, USA.

Received: 23 April 2013 Accepted: 1 August 2013
Published: 8 August 2013

References


Proteolytic enzyme combination reduces inflammation and oxidative stress and improves insulin sensitivity in a model of metabolic syndrome. Adv Enzyme Res. 2015.


Impact of systemic enzyme supplementation on low-grade inflammation in humans.
Pharma Nutr. 2015.
Impact of systemic enzyme supplementation on low-grade inflammation in humans

Marie-Eve Paradis, Patrick Couture, Iris Gigleux, Johanne Marin, Marie-Claude Vohl, Benoît Lamarche *

Institute of Nutrition and Functional Foods (INAF), 2440 Hochelaga Boulevard, Laval University, Que. G1V 0A6, Canada

ARTICLE INFO

Keywords:
- CRP
- IL-6
- Subclinical inflammation
- Systemic enzyme
- Proteolytic enzyme

ABSTRACT

Systemic enzyme therapy has been shown to be efficient in treating pain and inflammation associated with injury or musculoskeletal disorders. However, whether systemic enzyme supplementation also attenuates subclinical inflammation remains to be investigated.

In this randomized controlled trial, we investigated the impact of systemic enzyme supplementation on inflammatory gene expression as well as on markers of inflammation in 24 adult men and women with subclinical inflammation (serum C-reactive protein [CRP] levels >1 mg/L and <10 mg/L). Participants were supplemented with systemic enzymes (Wobenzym®450 FIP from bromelain and 1440 FIP from trypsin, 6 tablets/d) or placebo for periods of 4 weeks separated by a 4-week washout period.

Systemic enzyme supplementation had no impact on expression levels of whole blood cell inflammatory genes compared with placebo but significantly reduced serum IL-6 levels (p = 0.04). However, there was a significant sex × treatment interaction for IL-6 (p = 0.02) and CRP (p = 0.007). Specifically, both serum IL-6 and CRP concentrations were significantly reduced in men (p ≤ 0.03) but not in women (p ≥ 0.08).

This study suggests that short-term supplementation with systemic enzymes may attenuate subclinical inflammation, with perhaps greater effects among men than among women.

© 2015 Published by Elsevier B.V.

1. Introduction

Inflammation is being increasingly recognized as a key etiological factor in the development of atherosclerosis and subsequent cardiovascular disease (CVD) [1] and is frequently found co-segregating with obesity and metabolic syndrome [1-3]. C-reactive protein (CRP) has been used extensively as a non-specific marker of the acute phase response for decades [4]. Data have further shown that CRP is a powerful predictor of CVD outcomes in epidemiological studies [5]. Indeed, studies that have investigated the predictive value of subclinical CRP levels have been relatively consistent in showing that individuals with high serum CRP levels (≥3.0 mg/L) are at greater risk of CVD compared to individuals with lower (<1.0 mg/L) CRP levels, independent of gender and plasma cholesterol concentrations [6]. Other blood markers of active subclinical inflammation include monocyte chemotactic protein (MCP-1), adiponectin and interleukins (IL) such as IL-6 [7].

Systemic enzyme therapy, which involves the oral delivery of primarily proteolytic enzymes in combination with rutin and administered in the absence of food, has been recommended for many years for the treatment of pain and inflammation associated with musculoskeletal disorders, arthritis and post-surgery [8-10]. However, the impact of systemic enzyme supplementation on subclinical inflammation associated with metabolic syndrome and obesity is less known. In rabbits fed a lipid-rich, metabolic syndrome-inducing diet for 8 weeks, supplementation with systemic enzymes significantly reduced serum CRP concentrations [11]. To the best of our knowledge, no study has yet documented the impact of systemic enzyme supplementation on subclinical inflammation in humans.

The objective of this study was to examine the impact of systemic enzyme supplementation on inflammatory gene expression in whole blood cells and on blood markers of inflammation in men and women with subclinical inflammation. We hypothesized that systemic enzyme supplementation for 4 weeks down-regulates the expression of genes associated with inflammation.

* Corresponding author. Tel.: +1 418 656 2131x4355; fax: +1 418 656 5877. E-mail address: benoit.lamarche@inaf.ulaval.ca (B. Lamarche).

http://dx.doi.org/10.1016/j.phanu.2015.04.004
2213-4344/© 2015 Published by Elsevier B.V.

Please cite this article in press as: M.-E. Paradis, et al., Impact of systemic enzyme supplementation on low-grade inflammation in humans, PharmaNutrition (2015), http://dx.doi.org/10.1016/j.phanu.2015.04.004
in whole blood cells and reduces the concentrations of inflammatory biomarkers.

2. Material and methods

2.1. Study design

The study was conducted as a double blind, crossover, randomized, placebo controlled trial at the Institute of Nutrition and Functional Foods (INAF) in Québec City, Canada. Participants were supplemented with systemic enzymes in the form of Wobenzym® or placebo for periods of 4 weeks each in random order, with a 4-week washout between the two treatment phases. Treatment sequence was assigned to participants via the use of random sequence of numbers. Allocation to treatment sequence was concealed by a secure computer-assisted method enabling preservation of assignments until enrollment was confirmed. The study sponsor held the trial codes, which were disclosed after completion of the statistical analyses. Study products (Wobenzym® and placebo) were supplied by Mucos Pharma GmbH & Co. KG (Oberhaching, Bavaria, Germany). The systemic enzyme product was delivered in tablets each providing 90 mg (450 FIP units) bromelain from pineapple, 48 mg (1440 FIP units) trypsin from bovine and porcine pancreas, and 100 mg rutin from Sophora japonica. The placebo contained no active ingredients. Both the enzyme product and placebo contained the same inactive ingredients, were enteric coated, and were white film coated to ensure blinding (titanium dioxide) (Table 1). Participants were instructed to consume 6 tablets/day, 45 min before a meal for 4 weeks. The study protocol was approved by Université Laval’s Research Ethics Board and is registered at ClinicalTrial.gov # NCT01848808.

2.2. Subjects

Men and women were recruited from the general population in the Québec City metropolitan area through paper advertisements and electronic newsletters. To be eligible, participants needed to be aged between 18 and 75 years and have serum CRP levels >1 mg/L and <10 mg/L on 2 separate days at screening. Exclusion criteria were: hypersensitivity to components of the systemic enzyme supplement, severe congenital or acquired coagulation disorders (e.g. hemophilia, in dialysis patients) or liver damage, pregnancy or breastfeeding, planned surgical operations during the study, any clinical signs or laboratory evidence for severe inflammatory, endocrine, renal/pulmonary, neurological, cardiovascular, metabolic, gastrointestinal, hematological, or psychiatric condition and active malignancy of any type other than basal cell carcinoma. Other exclusion criteria were current use of anticoagulants or platelet aggregation inhibitors, chemotherapy agents, antibiotics, medication for lipids, diabetes, hypertension, inflammation, autoimmune diseases, mood disorders or NSAID within 1 month of entering the study, excessive alcohol consumption (more than two drinks per day for men, one for women) or alcoholism, smoking, drug use and history of drug abuse, as well as current use of supplements or natural health products.

A total of 250 subjects were screened by phone and 91 of them were invited to a first screening visit. Forty-one potentially eligible subjects, based on a first serum CRP level of >1 mg/L, were invited to the 2nd visit to complete the screening process, including a second assessment of serum CRP. A total of 27 subjects met all eligibility criteria (10 men and 17 women). One female interrupted her participation because of adverse event (gastritis). There were two other dropouts due to lack of availability during the study. Thus, 24 subjects completed the study (see study flow chart at Fig. 1).

2.3. Measurements

Subjects were instructed to avoid intense physical exercise 36 h before blood samples were taken and came to the clinical investigation unit after a 12 h overnight fast. Inflammatory gene expression in whole blood cells was assessed on samples collected at the end of each treatment phase. Serum concentrations of inflammatory markers and lipid levels were measured twice on two consecutive days after each treatment. The mean of the two post-treatment measurements was used in the analyses. General health assessment (complete blood count, liver and kidneys function), blood pressure, anthropometric measurements (height, weight, waist and hip girths, and body composition) as well as medical history were assessed prior to randomization. Participants also completed a questionnaire assessing diet and physical activity, as well as occurrence of any side effects during the study, as detailed below.

2.3.1. Anthropometry and blood pressure

Anthropometric measurements (body weight, height, waist and hip girths) were collected according to standardized procedures [12] at the first screening visit as well as before and after each phase. Systolic and diastolic blood pressures were averaged from 3 measurements taken after a 10 min rest in the sitting position using an automated blood pressure monitor (Omron, HEM-907XL).

2.3.2. Body composition assessed with dual-energy X-ray absorptiometry (DXA)

Baseline body composition was measured prior to initiating the first treatment phase with dual-energy X-ray absorptiometry (GE Lunar Prodigy Advance, GE Lunar Corporation, Madison, WI, USA). The scanner was calibrated before each measurement session against the standard calibration block supplied by the manufacturer for possible baseline drift. A quality-control test to monitor the reproducibility and stability of data was also performed before each session using a spine phantom provided by the manufacturer. The value from the quality-control test was plotted on graphs, and the score of each measurement was required to be within ± 0.05 g/cm² of the baseline result. More detail on the procedure is provided in Supplementary material.

2.3.3. Diet and physical activity

Eligible subjects received instructions from a registered dietitian regarding the forbidden use of specific supplements and medication during the study and to keep their nutritional and physical activity habits constant. Dietary intake during the study was assessed on three occasions using a validated web-based food-frequency questionnaire (FFQ) [13]: 1- at study entry; 2- after the first treatment and 3- after the second treatment. This validated FFQ inquires on food intake over the last 4 weeks, which is consistent with treatment duration in this study. Data from these questionnaires were analyzed using the Nutrient Data System software based on a mix of Canadian and FDA-produced nutrient...
2.3.4. Pro- and anti-inflammatory gene expression measurement

PAXgene blood RNA kits (Qiagen, Mississauga, ON, Canada) were used to isolate mRNA from whole blood cells taken on the last day of each treatment phase. The concentration of the purified RNA was analysed using NanoDrop (Thermo Scientific, Wilmington, DE, USA). Complementary DNA (cDNA) synthesis was completed using 1 μg of total RNA and the High Capacity cDNA Kit (Life Technologies, Foster City, CA, USA). Gene expression was assessed by real-time PCR (RT-PCR) using Applied Biosystems Gene Expression Assays. Primers and TaqMan® probes were obtained from Applied Biosystems (interleukin-1beta (IL-1β): Hs01555410_m1, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NfκB1): Hs00765730_m1, peroxisome proliferator-activated receptor alpha (PPARA): Hs00947936_m1, tumor necrosis factor (TNF): Hs00174128_m1, TNF-receptor associated factor 3 (TRAF3): Hs00936781_m1). Applied Biosystems, Foster City, CA, USA). Each sample was analyzed in triplicate and calibrated to the ATCB housekeeping gene (Hs99999903_m1). Relative quantification was performed on an Applied Biosystems 7500 Real, Time PCR System and the ΔΔCT calculation method was used to assess the mean fold expression difference (MFED) between the two treatments [15].

2.3.5. Pro- and anti-inflammatory markers and serum lipids

Serum CRP levels were measured using the Behring Latex-Enhanced (highly sensitive) CRP assay on the Behring Nephelometer BN-100 (Behring Diagnostic, Westwood, MA) and the calibrators (N Rheumatology Standards SL) provided by the manufacturer as described previously [16]. Serum adiponectin was measured by ELISA (B-Bridge International Inc., #K1001-1). Serum IL-6 was assessed by immunoassay (R&D system, #HS600B). Monocyte chemoattractant protein-1 (MCP-1) was assessed by immunoassay (R&D system, #DCP00). Inter-assay coefficients of variations (CV) for each of these assays were <1% (CPR), 5.2% (adiponectin), 7.8% (IL-6) and 5.7% (MCP-1). Serum cholesterol (C), triglycerides and HDL-C were assessed on a Roche/Hitachi Modular according to the manufacturer's specifications and using proprietary reagents. Plasma LDL-C concentrations were calculated using the Friedewald Equation. CVs for cholesterol, LDL-C, TG and HDL-C were all below 3%.

2.4. Safety and intolerance symptoms

Complete blood count, blood clotting and liver and kidney functions were assessed after each of the 4-week supplementation periods. Subjective tolerance ratings of the frequency and intensity of side effects were obtained by questionnaire administered on site at the beginning and end of each treatment period. On each occasion, participants indicated whether each side effect was absent (0), of mild intensity (1), of moderate intensity (2) or of severe intensity (3).

2.5. Statistical analyses

Statistical analyses were undertaken in a blinded fashion without knowledge of treatment allocation. The primary analyses consisted in comparing the expression levels of anti- and pro-inflammatory genes in whole blood cells between systemic enzyme supplementation and placebo. The secondary analyses consisted in assessing the change in the serum inflammatory markers and lipid levels with treatment. Data were analyzed using the PROC MIXED procedure for repeated measures in SAS (version 9.3; SAS, Inc., Cary, NC). Treatment and sex were considered as fixed effects and subject was considered as random effect. Interaction of treatment by sex was investigated systematically for each study outcome by introducing the appropriate term into the mixed models. There was no significant treatment by sequence interaction for any of the study outcomes. Variables were log-transformed if needed prior to statistical analysis. In such cases, geometric means are presented. Differences have been considered significant at $P \leq 0.05$ (two-tailed). Analyses were undertaken on a
per protocol basis, i.e. only in subjects with complete data (N=24), excluding dropouts.

3. Results

3.1. Subject characteristics at baseline

Table 2 shows the baseline characteristics of the 15 women and 9 men who completed the study. Supplemental Table 1 (Supplementary material) shows their physical activity levels as well as usual dietary habits. Mean age (S.D.) of participants was 45.5 (17.1) years. Six of the women were postmenopausal. With respect to ethnicity, 23 subjects were Caucasian and 1 was Asian. All participants were non-smokers. Mean systolic and diastolic blood pressures, blood lipids and glucose status were in the normal range. Average serum CRP concentration at screening was 2.99 (1.90) mg/L and was slightly higher in women than in men.

3.2. Systemic enzyme supplementation and inflammation

Table 3 summarizes the impact of 4-week systemic enzyme supplementation vs. placebo on whole blood expression levels of inflammatory genes. There was no difference between treatments in the expression of any of the selected genes. As shown in Table 4, 4-week supplementation with systemic enzymes vs. placebo significantly reduced serum IL-6 concentrations (p = 0.04) but had no effect on mean serum CRP (p = 0.47), MCP-1 (p = 0.39) and adiponectin levels (p = 0.73). A significant treatment by sex interaction was observed for CRP (p = 0.007) as well as for IL-6 (p = 0.02). As shown in Fig. 2, 4-week supplementation with systemic enzymes compared with placebo significantly reduced serum CRP (p = 0.03) and IL-6 (p = 0.008) in men but not in women (p ≥ 0.08).

3.3. Systemic enzyme supplementation and blood lipids

As shown in Table 5, there was no effect of systemic enzyme supplementation on lipid levels, with the exception of serum cholesterol, which was increased slightly but significantly by 3.1% (P = 0.05). Participants’ weight, waist circumference and blood pressure remained unchanged with treatments (not shown).

Table 3

<table>
<thead>
<tr>
<th>Gene</th>
<th>Systemic enzymes</th>
<th>Placebo</th>
<th>Fold change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>7.07 (0.14)</td>
<td>7.04 (0.22)</td>
<td>0.98</td>
<td>0.82</td>
</tr>
<tr>
<td>NF-κB</td>
<td>6.89 (0.08)</td>
<td>6.83 (0.13)</td>
<td>0.96</td>
<td>0.50</td>
</tr>
<tr>
<td>PPAR-α</td>
<td>9.47 (0.09)</td>
<td>9.43 (0.12)</td>
<td>0.97</td>
<td>0.70</td>
</tr>
<tr>
<td>MCP-1</td>
<td>8.60 (0.09)</td>
<td>8.61 (0.12)</td>
<td>1.01</td>
<td>0.92</td>
</tr>
<tr>
<td>TRAF3</td>
<td>8.16 (0.08)</td>
<td>8.17 (0.15)</td>
<td>1.01</td>
<td>0.87</td>
</tr>
</tbody>
</table>

Values are presented as means (standard error of the means). Each gene expression was calibrated to the ATCB housekeeping gene. Higher values indicate lower gene expression.

Analyses presented there are based on the PROC MIXED procedure in SAS on N=24 subjects. P value from the main effect of treatment in the mixed model. Abbreviations: IL-1β, interleukin-1β; NF-κB, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1; PPAR-α, peroxisome proliferator-activated receptor alpha; TNF, tumor necrosis factor; TRAF3, TNF-receptor associated factor 3.

3.4. Compliance and side effects

Compliance as assessed by number of tablets returned to the research team was very high (95%) and was comparable between the two treatments (not shown). There was no difference between the two treatments on safety parameters assessed (liver and kidney functions, Supplemental Table 2). There was no difference between treatments in number of red cells, white cells and platelets. There was a significant reduction in the absolute count of lymphocytes after systemic enzyme supplementation (P = 0.03) while the relative count of lymphocytes remained unchanged (P = 0.07, Supplemental Table 3). Occurrence of side effects was low and similar between the two treatments, with the exception of fatigue intensity rating and frequency, which was slightly higher during systemic enzyme supplementation than during placebo (p = 0.04 both for intensity and frequency, not shown).

4. Discussion

Several studies on inflammation related to arthritis and post-surgery have reported beneficial anti-inflammatory effects of supplementation with systemic enzymes. Less is known about this treatment modality on subclinical inflammation associated with obesity and metabolic syndrome. Our hypothesis was that supplementation with systemic enzymes attenuates inflammation in men and women with subclinical inflammation and that this effect can be perceived at the gene expression level as well as at the

Table 4

<table>
<thead>
<tr>
<th>Inflammation variables</th>
<th>Systemic enzymes</th>
<th>Placebo</th>
<th>% Difference</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP (mg/L)</td>
<td>2.17 (2.45)</td>
<td>2.16 (2.13)</td>
<td>0.5%</td>
<td>0.47</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>1.25 (0.89)</td>
<td>1.41 (0.98)</td>
<td>–11.3%</td>
<td>0.04</td>
</tr>
<tr>
<td>MCP-1/CCL2 (pg/mL)</td>
<td>367.6 (113.1)</td>
<td>356.3 (100.7)</td>
<td>3.1%</td>
<td>0.39</td>
</tr>
<tr>
<td>Adiponectin (μg/mL)</td>
<td>8.43 (1.53)</td>
<td>8.53 (1.50)</td>
<td>–1.2%</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Values are presented as means (S.D.) and percentage of change from placebo. Analyses presented there are based on the PROC MIXED procedure in SAS on N=24 subjects. Abbreviations: CRP, C-reactive protein; IL-6, interleukin-6; MCP-1/CCL2, monocyte chemotactic protein.

* P value from the main effect of treatment in the mixed model. Models for CRP and IL-6 included treatment, sex and treatment by sex interaction; Model for MCP-1/CCL2 included treatment only; model for adiponectin included treatment and sex.
* Analyses performed on log-transformed values. Geometric means are presented in such cases.

Please cite this article in press as: M.-E. Paradis et al., Impact of systemic enzyme supplementation on low-grade inflammation in humans, PharmaNutrition (2015), http://dx.doi.org/10.1016/j.phanan.2015.04.004
level of blood biomarkers of inflammation. Data suggest that supplementation with a mix of systemic enzymes (Wobenzym®) for 4 weeks has no effect on expression levels of genes related to inflammation in whole blood cells. However, systemic enzyme supplementation may have subclinical anti-inflammatory effects, as evidenced by a reduction in serum IL-6 concentrations. Although our study was not originally designed specifically to investigate differences between men and women, data revealed a significant treatment by sex interaction for IL-6 and CRP concentrations. Specifically, reduction in both of these inflammatory markers was significant in men but not in women.

While previous studies have investigated systemic enzymes in acute inflammatory conditions have shown similar anti-inflammatory effects in both men and women [8,17], other evidence have highlighted sex differences in inflammation processes and levels [18–20]. Sex differences in CRP concentrations may be due at least partly to the impact of estrogens as premenopausal women have higher levels of CRP compared to men [18] and hormone replacement therapy has also been associated with increased CRP concentrations [21,22]. Data from our sample are compatible with this concept that women tend to have higher serum CRP levels than men (+42% higher CRP in women compared with men). Recent evidence has also suggested that both gene expression and inflammatory biomarkers may respond differently to dietary change [23,24]. Further studies are therefore needed in a context of subclinical inflammation to validate the observation that men may be more responsive than women to certain anti-inflammatory therapies, such as systemic enzyme supplementation.

The apparent disconnect between the absence of change in inflammatory gene expression and the change in inflammatory marker levels in men also needs to be discussed. Changes in gene expression induced by a dietary intervention generally occur rapidly, before changes in protein level can be observed. However, protein levels reflect the balance between both RNA and protein production and turnover. In that context, the correlation between the concentrations of proteins and their corresponding mRNAs is not always strong [25]. In the present study, systemic enzymes were supplemented for a period of 4 weeks, which should have been long enough to induce changes in gene expression levels, especially considering that small but significant anti-inflammatory effects were observed. It is possible that the effect of systemic enzymes on gene expression occurred acutely, i.e. after each supplementation of the tablets, and that it was not perceived when gene expression levels were measured at the end of the treatment period. It is also likely that the potential anti-inflammatory effect of systemic enzyme supplementation was not mediated by the particular genes and transcription factors investigated in this study, or that whole blood cells do not reflect the anti-inflammatory changes occurring in other tissues after supplementation with systemic enzymes.

Mechanisms through which systemic enzyme products such as Wobenzym® may have anti-inflammatory effects have been proposed [11,26,27]. Talaieva and Bratus speculated that systemic enzymes may act indirectly through reduction of inflammation-associated reactive oxygen species generation, and/or through the effect of other products in the formulation such as rutin, a flavonoid with antioxidant properties [28]. Others have suggested that these proteases may form complexes with cytokines through the interaction of specific anti-proteases widely available in human blood and promote their clearance from inflamed tissues via endocytotic and phagocytic routes [29,30]. Other mechanisms, such as interactions with cell surface receptors and signal transduction events have been suggested [29]. One of the systemic enzyme ingredients, bromelain, has been shown to have anti-inflammatory effects as evidenced by an attenuated IL-1β, IL-6 and TNF-α from THP-1 cells after LPS stimulation [31].

At the beginning and at the end of each treatment period, participants were invited to indicate the frequency and intensity of side effects over the last 4 weeks. Participants reported slightly but significantly more fatigue while being supplemented with systemic enzyme than placebo both for intensity and frequency. However, there was no difference in the frequency of other side effects between systemic enzyme supplementation and placebo. There was also no difference between the two treatments on safety parameters of liver and kidney function. The reduction in the absolute count of lymphocytes observed with the systemic enzyme treatment was significant but remained in the normal range. Moreover, the absolute count was not different and the white cells,

Fig. 2. Serum CRP and IL-6 levels in women and men after a 4-week supplementation with systemic enzymes (black bars ■) or placebo (open bars □). Values are presented as means (standard error of the means).

Table 5

Lipid profile after supplementation with systemic enzymes for 4 weeks vs. placebo supplementation.

| Lipid variables | Systemic enzymes | Placebo | % difference | p
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.61 (0.98)</td>
<td>5.44 (1.03)</td>
<td>3.1%</td>
<td>0.05</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>3.22 (0.74)</td>
<td>3.11 (0.77)</td>
<td>3.6%</td>
<td>0.17</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.74 (0.45)</td>
<td>1.71 (0.48)</td>
<td>1.2%</td>
<td>0.40</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)a</td>
<td>1.28 (1.55)</td>
<td>1.21 (1.59)</td>
<td>5.5%</td>
<td>0.20</td>
</tr>
<tr>
<td>Total cholesterol/HDL-C ratio</td>
<td>3.40 (0.93)</td>
<td>3.37 (1.00)</td>
<td>0.9%</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Values are presented as means (S.D.) unless stated otherwise. Analyses presented there are based on the PROC MIXED procedure in SAS on N = 24 subjects. Abbreviation: C, cholesterol.

a p value from the main effect of treatment in the mixed model. Models included treatment only.

b Analyses performed on log-transformed values. Geometric means are presented.
platelets and hemoglobin counts remained similar. This is consistent with previous data and a long history of use having established that systemic enzymes such as Wobenzym® are considered safe.

This study has strengths and limitations. The crossover randomized double-blind design of this study is a major strength. Statistics were also performed in a blinded fashion, reducing the risk of biases. Markers of inflammation were measured twice at screening and after each treatment phase, which contributed to reduce intra-individual variability and hence increased statistical power. Compliance was very high (>95%), with only one participant with a compliance below 80% (79.8%). The number of subjects who completed the study was high (24/27). This is to the best of our knowledge the first study to have documented the side effects and the safety of a chronic use (4 weeks) of systemic enzymes in healthy volunteers with subclinical inflammation. On the other hand, the sample size was limited and not specifically calculated to investigate potential treatment by sex interaction in the response to treatment. The study design did not allow us to investigate the impact of systemic enzyme supplementation on inflammation in an acute setting, i.e. immediately after ingestion of the supplement.

In conclusion, data from this study suggest for the first time that short-term supplementation with systemic enzymes may attenuate inflammatory processes in healthy individuals with subclinical inflammation, and that this effect may be more important in men than in women. This apparent difference in inflammatory responses to systemic enzyme supplementation between men and women needs to be further assessed in future studies.

5. Disclosure

This study was supported through funding from Atrium Innovations. Study products were provided by Mucos Pharma GmbH & Co. KG (Oberhaching, Bavaria, Germany). Sponsors had no role to play in the running of the study, in data analysis and in decision to publish this manuscript.

Acknowledgments

We are grateful to the participants, without whom the study would not have been possible. We also express our gratitude to Steeve Larouche and Christiane Landry of the Institute of Nutrition and Functional Foods for their technical assistance and for the expert care provided to the participants. Benoît Lamarche is Laval University Chair of Nutrition. Marie-Claude Vohl is a Tier 1 Canada Research Chair in Genomics Applied to Nutrition and Health.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phanu.2015.04.004.

References


Please cite this article in press as: M.-E. Paradis, et al., Impact of systemic enzyme supplementation on low-grade inflammation in humans, PharmaNutrition (2015), http://dx.doi.org/10.1016/j.phanu.2015.04.004
Proteolytic enzyme combination reduces inflammation and oxidative stress and improves insulin sensitivity in a model of metabolic syndrome.
Adv Enzyme Res. 2015.
Proteolytic Enzyme Combination Reduces Inflammation and Oxidative Stress and Improves Insulin Sensitivity in a Model of Metabolic Syndrome

Tetiana V. Talaieva*, Victor V. Bratus

National Scientific Center, ND Straschesko Institute of Cardiology, Academy of Medical Sciences of Ukraine, Kiev, Ukraine
Email: talaieva-t@yandex.ru

Received 6 February 2015; accepted 17 February 2015; published 2 March 2015

Copyright © 2015 by authors and Scientific Research Publishing Inc.
This work is licensed under the Creative Commons Attribution International License (CC BY).

http://creativecommons.org/licenses/by/4.0/

Abstract

Chronic, low-level inflammation may be an independent marker of Metabolic Syndrome (MetS). Systemic Enzyme Therapy (SET), the oral administration of proteolytic enzymes, is safe and effective in the management of inflammation. Therefore, the effects of SET, as Wobenzym®, on the prevention and treatment of inflammation and other metabolic risk factors were assessed in a rabbit model of diet-induced MetS. Animals were fed a lipid-enriched diet for 8 weeks during which they were administered a vehicle control (control group) or Wobenzym either throughout the study period (prevention group) or beginning at the 5th week, after the development of biomarkers of MetS (treatment group). At the 8th week, both prevention and treatment groups demonstrated improved insulin sensitivity relative to the control group and reduced serum C-reactive protein (CRP) and glycosylated hemoglobin (HbA1c, \( P < 0.001 \)). At 8 weeks, the prevention group, but not the treatment group, exhibited reduced total cholesterol and oxidative stress, measured as serum malondialdehyde (\( P < 0.001 \)). Triglycerides and free fatty acids were reduced in both the treatment (\( P < 0.01 \)) and prevention groups (\( P < 0.001 \)) relative to the control group at the 8th week. Body weight and blood glucose were not affected. Enzyme therapy may have a positive effect on inflammation, insulin sensitivity, and other metabolic risk factors of MetS.

Keywords

Metabolic Syndrome, Systemic Enzyme Therapy, Wobenzym, Protease, Inflammation, CRP

*Corresponding author.
1. Introduction

Metabolic Syndrome (MetS) is generally characterized by obesity and the presence of two additional metabolic risk factors [1]. More specifically, the risk factors encompassing MetS include abdominal obesity (waist circumference ≥ 40 inches for men and ≥35 inches for women), impaired fasting blood glucose (≥100 mg/dL), elevated triglycerides (≥150 mg/dL), low high-density lipoprotein (HDL < 40 mg/dL for men and <50 mg/dL for women), and elevated blood pressure (≥130/85 mmHg) [1]. A public health crisis, MetS affects an estimated 20% - 34% of the population [2], increasing the risk for cardiovascular disease, stroke and diabetes and driving up medical costs. In the US alone, the health care costs associated with cardiovascular disease, obesity and diabetes are estimated at $444 billion, $147 billion, and $218 billion, respectively [2]-[4]. Although largely preventable and modifiable by nutrition and lifestyle interventions, few pharmaceutical or nutritional agents are available to address the complex, multiple risk factors that contribute to MetS.

Emerging data suggest that MetS is associated with both inflammation and oxidative stress and that these may be independent risk factors for diabetes, hypertension and cardiovascular events, even after adjustment for established risk factors such as dyslipidemia [5]-[8]. It has further been proposed that C-reactive protein (CRP), as a marker of general inflammation, may be an independent predictor of long-term cardiovascular risk and that targeting elevated CRP might reduce the incidence of cardiovascular events [7] [9]-[10]. Statin therapy has been debated as a treatment for elevated CRP, independent of low-density lipoprotein (LDL) cholesterol levels and as primary prevention in patients at intermediate risk for heart disease [7] [9]. However, statin therapy is associated with myotoxicity and an increased incidence of diabetes [9].

Systemic Enzyme Therapy (SET) is the oral administration of combinations of proteolytic enzymes from plant sources, such as bromelain (Ananas comosus fruit) and papain (Carica papaya fruit); bovine or porcine pancreatic enzymes, such as trypsin, chymotrypsin, and pancreatin; and in some preparations, fungal-sourced lipase and amylase. Wobenzym® also includes the bioflavanoid rutin, which has been demonstrated to reverse and prevent metabolic changes such as abdominal fat stores, glucose tolerance, hepatic and cardiovascular function, as well as attenuate oxidative stress and inflammation in a rat model of MetS [11]. Systemic enzymes have a long history of use as natural anti-inflammatory agents, and such products are available commercially as dietary supplements or pharmaceuticals. A recent review summarizes the absorption and pharmacokinetics of orally administered proteolytic enzymes and provides the rationale behind the anti-inflammatory effects of enzyme-antiprotease complexes [12]. Numerous animal [13]-[16] and human trials [17] [18] have demonstrated the clinical benefits of systemic enzyme therapy in inflammatory conditions, most commonly in comparative trials concluding non-inferiority to non-steroidal anti-inflammatory drugs (NSAIDs) [19], as well as in reducing general inflammation measured as C-reactive protein [20] [21]. However, to our knowledge, SET has not been studied in inflammation as related to parameters of MetS. Therefore, a pre-clinical trial was conducted to determine the effects of orally administered proteolytic enzymes on the prevention and treatment of inflammation, insulin sensitivity, oxidative stress, and other metabolic markers in an animal model of diet-induced MetS.

2. Materials and Methods

2.1. Animals and Diets

Male and female Chinchilla rabbits, aged 3 to 4 months, were obtained from the Ukranian Academy of Medical Sciences. The animal protocol was approved and housing provided by the National Scientific Center, ND Strachesko Institute of Cardiology in Kiev. The investigation included 50 rabbits with baseline body weights of 2.6 ± 0.15 kg that were fed a regular diet supplemented with 0.75 g/kg of body weight per day of dry milk cream containing 80% saturated fatty acids for 8 weeks with free access to water. The rabbits were separated into 3 groups: control group (n = 30), prevention group (n = 10), and treatment group (n = 10). Animals in the control group were fed the high-fat diet and received a saline vehicle control. Animals in the prevention group were fed the high-fat diet and administered Wobenzym® (Mucos Pharma GmbH, Oberhaching, Germany) for the full 8 weeks. Animals in the treatment group were fed the high-fat diet and administered Wobenzym from the 5th week to the 8th week. Wobenzym was dosed at 1 tablet per 6 kg of body weight, based on preliminary tests and equivalent to the typical human dose by weight. Tablets were ground and delivered in sterile saline directly into the small intestine via gastric tube connected to a Janne syringe.
2.2. Blood Collection and Parameters

Blood samples were obtained at baseline and weeks 2, 4, 6, and 8 from the marginal ear vein, centrifuged at 4000 RPM, and processed immediately without freezing, as previously described [22]. The determinations of total cholesterol, triglycerides (Tg), free fatty acids (FFA), blood glucose, glycosylated hemoglobin (HbA1c), and CRP were carried out using standard BioSystem test kits according to the manufacturer’s protocols (Spain) and analyzed on a BTS-330 biochemical analyzer (Spain). Lipid oxidation was determined as the plasma malondialdehyde concentration using a standard reaction with thiobarbituric acid, TBARS (Sigma, USA).

2.3. Insulin Sensitivity

Systemic insulin sensitivity was determined as changes in plasma glucose level as a percentage of the initial value 60 min after subcutaneous insulin injection (Actrapid® HM, Novo Nordisk, Denmark), as previously described [23]. Results are reported as percent glucose clearance, calculated as follows:

\[
\% \text{ Glucose Clearance} = \left( \frac{\text{glucose at baseline}}{\text{glucose at 60 min}} \right) \times 100.
\]

2.4. Statistical Analyses

Data are presented as the mean ± SEM. Analyses were performed using GraphPad InStat 3 software (La Jolla, CA). Comparisons between and within groups were made by analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons. Mann-Whitney U tests are used when the data are not normally distributed. Significance was accepted at \( P < 0.05 \).

3. Results

3.1. Lipid Loading Induced Insulin Resistance and Metabolic Alterations

Chronic lipid loading over 8 weeks induced a deterioration of systemic insulin sensitivity, determined as a reduction in percent glucose clearance following subcutaneous insulin injection (Figure 1(a), \( P < 0.001 \)). Lipid loading was also associated with increased inflammation (Figure 1(b), \( P < 0.001 \)), oxidative stress (Figure 1(c), \( P < 0.001 \)), HbA1c (Figure 1(h), \( P < 0.001 \)), and increased in lipid levels (total cholesterol, total TG and FFA levels (Figure 1(d)-(f), \( P < 0.001 \)) compared to baseline values.

3.2. Systemic Enzyme Therapy Prevented the Decrease in Insulin Sensitivity

Systemic insulin sensitivity was determined as changes in blood glucose levels as a percentage of the initial value at 60 min following subcutaneous insulin injection. At baseline, glucose clearance was 54.4% ± 0.5%, but in lipid-fed control animals this decreased to 8.0% ± 0.9% at the 8th week (Figure 1(a)). The prevention and treatment groups maintained glucose clearance at 30.4% ± 2.6% and 23.2% ± 2.5%, respectively, which differed from the control group at the 8 week (\( P < 0.001 \)).

3.3. Systemic Enzyme Therapy Reduced Diet-Induced Inflammation

Lipid loading raised serum CRP throughout the study period from 1.75 ± 0.11 mg/L at baseline to 13.6 ± 0.74 mg/L and 32.03 ± 2.22 mg/L in the control group at the 4th and 8th weeks, respectively (Figure 1(b)). At the 4th week, the increases in serum CRP in both the prevention (10.1 ± 1.4 mg/L) and treatment (13.4 ± 2.49 mg/L) groups were similar to control. At week 6, however, serum CRP levels in the prevention group (11.4 ± 1.15 mg/L) but not in the treatment group (19.2 ± 2.54 mg/L) were significantly lower from control group (25.0 ± 1.25 mg/L) (\( P < 0.001 \)). Serum CRP decreased in week 8 compared to week 6 in both the prevention (6.3 ± 0.38 mg/L) and treatment (11.34 ± 1.41 mg/L) groups, and both groups differed from control (\( P < 0.001 \)).

3.4. Systemic Enzyme Therapy Prevented Oxidative Stress

Oxidative stress, measured as serum malondialdehyde, increased in both the control and treatment groups as compared to baseline (Figure 1(c)). However, concurrent SET prevented the increase in oxidative stress observed with chronic lipid loading (\( P < 0.001 \)). Treatment with SET initiated at the 5th week, did not affect oxida-
3.5. Systemic Enzyme Therapy Mediated Dyslipidemia

Chronic lipid loading increased total cholesterol, TG, and FFA in all groups compared to baseline (Figure 1(d)-(f)). Prevention and treatment groups did not differ from the control group for cholesterol, TG, and FFA through 6 weeks. At week 8, total cholesterol was reduced in the prevention group (1.28 ± 0.02 mmol/L) com-
pared to control group (2.32 ± 0.14 mmol/L) (Figure 1(d), \( P < 0.001 \)). Triglycerides were significantly lower in both the prevention (1.32 ± 0.03 mmol/L, \( P < 0.001 \)) and treatment (1.82 ± 0.08 mmol/L, \( P < 0.01 \)) groups compared to control (2.49 ± 0.15 mmol/L) at week 8 (Figure 1(e)). Similarly, FFA were lower in the prevention (\( P < 0.001 \)) and treatment (\( P < 0.01 \)) groups compared to the control group at week 8 (Figure 1(f)). Free fatty acids appeared elevated in the treatment group at week 6 but this was not significant. In all three parameters, the treatment group appeared to follow the control group until week 6 (treatment initiated at week 5) and then declined relative to control.

3.6. Systemic Enzyme Therapy Did Not Affect Blood Glucose but Reduced HbA1c

Fasting blood glucose increased in response to lipid loading from 7.34 ± 0.46 mmol/L at baseline to 13.8 ± 0.81 mmol/L at the 8th week in the control group (Figure 1(g)). Although blood glucose levels appeared to be reduced in both the prevention (10.8 ± 0.74 mmol/L) and the treatment (10.2 ± 0.27 mmol/L) groups compared to control, the differences were not significant. However, while lipid loading significantly increased in HbA1c throughout 8 weeks in the control group, concurrent SET prevented the increase in HbA1c in the prevention group at all time points (3.54 ± 0.17 vs. 10.27 ± 0.61 µmol fructose per 1g Hb at week 8, \( P < 0.001 \)). Enzyme treatment initiated at week 5 also lowered HbA1c relative to control, observed at both the 6th (\( P < 0.01 \)) and 8th weeks (Figure 1(h), \( P < 0.001 \)).

4. Discussion

Hyperglycemia, impaired glucose disposal, hyperlipidemia, inflammation, and oxidative stress are central features of MetS. However, a limited etiological comprehension, together with a lack of consensus on qualitative and quantitative clinical definitions, has complicated the development and validation of an animal model that faithfully recapitulates human MetS.

While the most widely utilized MetS models are mice, rabbits offer a potentially valuable tool as they can depict a broader landscape of diet-induced MetS pathologies over a longer period of time. Their use in over 1000 studies of diabetes to date supports their basic utility in examining insulin sensitivity [24]. In response to fat and cholesterol feeding, rabbits develop the most well-established MetS features, including systemic inflammation, glucose intolerance, hyperlipidemia, hypertension, accrual of visceral fat tissue, and fatty liver disease [25]-[32]. Relative to mice, the lipoprotein profile of rabbits also corresponds more closely to humans, and in contrast to mice, which exhibit an atheroprotective phenotype, rabbits with a constellation of MetS features develop coronary atherosclerotic lesions resembling that of humans [24] [26]. In addition, as rabbits exhibit a longer life span of 5 - 8 years, lengthier study durations may afford more extensive interrogation of MetS and its complications in an easily handled, inexpensive animal model.

The Chinchilla rabbit employed in the current study corresponds, in baseline biochemical parameters, age, and approximate weight, with other rabbit models used to investigate MetS. Here, we report baseline fasting glucose, total cholesterol and Tg levels that are similar to published values from a recently developed rabbit model of high-fat diet (HFD)-induced MetS [25] [31] [32]. Baseline CRP values were similar to those reported in Japanese White rabbits utilized to investigate MetS [29]. The plasma response of glucose to insulin injection in the fasting state was consistent with published data using the same insulin preparation, dose and mode of administration [33].

The administration of SET, either as prevention beginning at baseline or as treatment beginning at the 5th week, failed to significantly reduce fasting glucose levels (Figure 1(g)). However, increased glucose clearance was evident in both groups (Figure 1(a)). Several potential reasons may account for this finding. The magnitude of improvement in insulin sensitivity may have been insufficient or of limited relevance to the fasting glucose level. Further, fasting glucose may have been somewhat refractory to the effects of insulin sensitization as a result of increased hepatic glucose production in the overnight fast. The lower HbA1c in SET-treated animals suggests that SET was attenuating blood glucose at time points other than the morning hours, such during the daytime postprandial periods.

While HbA1c is universally employed to assess long-term glycemic control in humans, considerable species differences exist, and a paucity of literature addresses its ranges in rabbits. In the current study, HbA1c was lowest in the SET prevention group, with significance demonstrated at the 2nd week (Figure 1(h)). The superior efficacy in the prevention group, which was of longer duration than the treatment approach, is consistent with
the cumulative nature of this marker. The most striking finding was the reduction in HbA1c at the 6th week in the treatment group, which received the first dose of SET at the 5th week. This rapid change occurred despite no concomitant alteration of systemic insulin sensitivity or fasting blood glucose. It is clear that in our rabbit model, this marker is subject to change modification within a short period of time.

Elevated CRP reflects systemic inflammation, a highly pertinent feature of MetS. In rabbits, this marker is inducible by stimuli such as dietary lipid enrichment [29] and is repressed by anti-inflammatory and anti-hyperglycemic interventions [27]. Like humans, rabbits exhibit elevations of CRP in tandem with progression of MetS features including atherosclerosis [29]. We demonstrate an approximate 10-fold increase in response to a HFD (Figure 1(b)), which is similar to previously reported data in rabbits that developed other MetS features, including visceral adiposity and atherogenic lesions, over 22 weeks of HFD feeding [29].

Malondialdehyde (MDA), a thoroughly validated marker of oxidative stress, increased 7-fold over 8 weeks (Figure 1(c)). Since proteases are not recognized as potent antioxidants, it is rational to speculate that SET may act indirectly through reduction of inflammation-associated reactive oxygen species generation, and/or the effect is due to the co-formulation with rutin, a flavonoid antioxidant with potential utility in MetS [34]. The absence of a therapeutic effect may be explained by oxidative stress comprising an early, initiating event in MetS. As a nonsignificant decrease was apparent in the treatment group between the 6th and 8th week, continuation of SET over a longer duration may be necessary for antioxidant effects to become detectable.

The 3-fold elevation of Tg in untreated rabbits was similar to the 3.5-fold increase in another MetS HFD-fed rabbit model after 12 weeks (Figure 1(e)) [25]. The SET as a preventive or treatment measure resulted in lower Tg levels very late in the study. A late reduction, although significant only in the prevention group, was also evident in total cholesterol levels. In untreated animals, elevation of total cholesterol in our model was modest compared to other studies of HFD-fed rabbits [25] [28]. For both Tg and cholesterol levels, SET as a prevention produced the lowest values throughout the study, while treatment had a relatively weak effect (Figure 1(d)). The short duration of treatment with SET (beginning at the 5th week, with analysis at the 8th week) was likely insufficient, and the nonsignificant decline suggests that continuation may have produced further reductions. Previous studies of MetS in rabbits have administered the HFD over a longer time period of 12 - 22 weeks [25] [28] [29] [32].

Several possible explanations may account for the general observation of superior efficacy of prevention relative to treatment. Foremost, the longer duration of supplementation in the prevention group may account for the superior efficacy. A second possibility is that SET exerts its effects in the early, initiating stages of MetS, as oxidative events and inflammatory signals are causally involved in the onset of insulin resistance [35]. A third possibility is that visceral adiposity, although not assessed, is likely to have increased as described previously [25] [29] [32], increasing oxidative stress and inflammation in the first weeks of high-fat diet to a point that requires a higher dose of SET [29] [36]. Alternatively, late commencement of SET may have been insufficient to address a modified climate of substantial and multifocal metabolic deregulation. From a theoretical perspective, a therapy that is effective when introduced at a later stage of disease must target pathways of reversal or attenuation, and these may differ from pathways of causation. Accordingly, preventive agents are not always curative, and vice versa.

While previous research has documented the bioavailability and therapeutic efficacy of SET as an anti-inflammatory intervention, there is limited knowledge of its mechanisms of action [12] [13] [37]. It has been demonstrated that these proteases, as complexes with endogenous antiproteases, complex with cytokines and promote their clearance from inflamed tissue via endocytotic and phagocytic routes [13]. Other mechanisms, such as interactions with cell surface receptors and signal transduction events, have been suggested [13]. In contrast to more extensively studied natural anti-inflammatory agents, such as fish oil and polyphenols, which intersect established pathways with known relevance to MetS, it remains unknown how proteolytic enzymes relate to the onset and progression of MetS.

5. Conclusion

The current data contribute to an evolving body of literature supporting the potential of anti-inflammatory interventions in MetS prevention and therapy. A continuation of this work is clearly warranted, including validation of this potentially valuable animal model in the study of MetS. Wobenzym® improved insulin sensitivity and reduced CRP and other risk factors associated with MetS in this model. Further research is required to determine
whether anti-inflammatory interventions, and SET in particular, might affect MetS development and progression in humans.

Acknowledgements

The authors thank Barry W. Ritz, PhD, Kelly Heim, PhD, and Kelly Gibson for their assistance in preparing the manuscript for publication, including adaptation to English.

References


Characteristic Fibrosis. Cholesterol-Fed Rabbit as a Unique Model of Nonalcoholic, Nonobese, Non-Insulin-Resistant Fatty Liver Disease with Diabetes.

Experimental and Clinical Endocrinology & Diet Induced Lipid Metabolic Disorders and Insulin Resistance in Rabbit.


Gastrointestinal absorption and biological activities of serine and cysteine proteases of animal and plant origin: review on absorption of serine and cysteine proteases.

Review Article

Gastrointestinal absorption and biological activities of serine and cysteine proteases of animal and plant origin: review on absorption of serine and cysteine proteases

Gerhard Lorkowski

GL Pharma Consulting Research & Development (GL Pharma CR&D), D-82131 Gauting/Munich, Germany

Received December 19, 2011; accepted February 12, 2012; Epub February 28, 2012; Published March 15, 2012

Abstract: Research has confirmed that peptides and larger protein molecules pass through the mucosal barrier of the gastrointestinal tract. Orally administered serine and cysteine proteases of plant and animal origin also reach blood and lymph as intact, high molecular weight and physiologically active protein molecules. Their absorption may be supported by a self-enhanced paracellular transport mechanism resulting in sub-nanomolar concentration of transiently free protease molecules or, in a complex with anti-proteases, at higher concentrations. Data from pharmacokinetic investigations reveals dose linearity for maximum plasma levels of free proteases not unusual for body proteases and a high inter-individual variability. There is no interference with each other after oral administration of protease combinations, and absorption follows an unusual invasion and elimination kinetic due to slow velocity of absorption and a fast 100% protein binding to anti-proteases. Oral application of proteases leads to increased proteolytic serum activity and increased plasma concentrations of the corresponding anti-proteases. Their biological activity is determined by their proteolytic activity as free proteases on soluble peptides/proteins or cell surface receptors (e.g. protease activated receptors) and their activity in the complex formed with their specific and/or unspecific anti-proteases. The anti-protease-complexes, during immune reaction and injuries often loaded with different cytokines, are cleared from body fluids and tissue by receptor mediated endocytosis on hepatocytes and/or blood cells. Oral administration of enteric coated tablets containing proteolytic enzymes of plant and animal origin may be a safe method to stabilize, positively influence or enhance physiological and immunological processes during disease processes and in healthy consumers.

Keywords: Gastrointestinal absorption, serine and cysteine protease, proteinase, proteolytic enzyme activity, anti-protease, protease activated receptors

Introduction

Single orally administered proteolytic enzymes of plant and animal origin are widely used as a medical treatment for a variety of digestive, absorption and pancreatic disorders. Historically, porcine and bovine pancreatic enzymes have been the preferred form of supplementation for exocrine pancreatic insufficiency. Plant-based enzymes, such as bromelain from pineapple, also serve as effective digestive aids in the breakdown of proteins [1].

However, orally administered proteolytic enzyme combinations often supplemented with rutosid are widely used as an alternative or a supplementary treatment of different disease conditions such as acute and post surgical trauma, phlebitis, rheumatoid and osteoarthritis, as well as in an adjunctive therapy in cancer treatment [2, 3]. The terms proteolytic enzymes, proteases or proteinases are often synonymously used and the medical treatment is often described as systemic enzyme therapy, which implies a gastrointestinal absorption for being active in the organism by distribution in body fluids.

In the United States and in Europe various proteolytic enzyme preparations are available on the market. Their marketing status is different and varies from prescription drug to OTC or food supplementation. They are marketed either as
Review on absorption of serine and cysteine proteases

Table 1. Overview of active ingredients used in 3 different marketed preparations

<table>
<thead>
<tr>
<th>Drug Substance</th>
<th>Preparation 1</th>
<th>Preparation 2</th>
<th>Preparation 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromelain</td>
<td>90 mg = 900 F.I.P.-units</td>
<td>45 mg = 450 F.I.P.-units</td>
<td>133-178 mg = 800 units</td>
</tr>
<tr>
<td>Papain</td>
<td>–</td>
<td>60 mg = 328 F.I.P.-units</td>
<td>–</td>
</tr>
<tr>
<td>Pancreatin</td>
<td>–</td>
<td>100 mg = 300 Ph. Eur.-units proteinase</td>
<td>–</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>–</td>
<td>1 mg = 596 F.I.P.-units</td>
<td>–</td>
</tr>
<tr>
<td>Trypsin</td>
<td>48 mg = 24 μkat*</td>
<td>24 mg = 12 μkat*</td>
<td>–</td>
</tr>
<tr>
<td>Rutosid</td>
<td>100 mg</td>
<td>50 mg</td>
<td>–</td>
</tr>
</tbody>
</table>

*1 μkat is the amount of enzyme which turns over 1 μM of substrate per second under standard conditions. It corresponds to 60 F.I.P. – Units.

Proteolytic enzyme combinations are generally provided as enteric coated tablets for oral administration at dosages varying from 1 to 200 mg per tablet. The amount of proteases provided in the preparation is described by their F.I.P. activity (F.I.P.-units of the Fédération Internationale Pharmaceutique). One F.I.P.-unit is the amount of enzyme which is able to turn over 1 μmol of substrate in 1 minute under standard conditions. Due to enteric coating, degradation of the protein structure to single amino acids or peptides in the acidic environment of the stomach is reduced. If at all, the absorption of proteases from the gastrointestinal tract may be favoured in the small intestine. Table 1 shows the characteristics of three typical representatives of proteolytic enzyme combinations.

In general, proteases’ side effects are few. Complications typically arise from either excessive dosing or allergic reaction to bovine or porcine substances, papaya or pineapple. In the case of excessive dosing, transient gastrointestinal upset may result. To avoid hypersensitivity reactions, it is best to confirm a patient is not allergic to the given proteases’ source prior to use. Hyperuricosuria (excess uric acid in the urine) and hyperuricemia (excess uric acid in the blood) are associated with extremely high doses of exogenous pancreatic enzymes [6].

The lack of unwanted drug interactions and major side-effects as well as overall safety aspects, make orally administered proteolytic enzyme combinations an interesting tool for the management of acute and chronic inflammatory processes. Nevertheless, the clinical benefit is subject to controversy as the biological rationale and the mechanism(s) of action are widely unknown and far from being understood. Additionally, clinical trials performed in the last century did not always come to an end with clinically relevant and statistically significant results. Often an insufficient patient number and organizational or other insufficiencies such as only mild to moderate disease activity at inclusion of patients were identified as a cause. As there is also doubt on the absorption at all or at least on sufficient amounts of larger protein molecules and missing proof of tissue distribution, clinically relevant systemic effects in the treatment of diseases are not readily accepted. Nevertheless, protease complexes with their counterpart anti-proteases (often as acute phase proteins in animals) [7], as well as the specific proteolytic activity of proteases on specific receptors (e.g. protease activated receptors, PAR) in the gastrointestinal tract [8] and, provided they are absorbed, in body fluids, may play a major role in many regulatory processes in humans [9, 10]. Thus, clinical effects and benefits are still a matter of controversy and clinical and pharmacological effects have to be further evaluated.

This article summarizes the existing data on gastrointestinal absorption of proteases after
oral administration and makes a mechanism of absorption reasonable. The pharmacokinetic data are summarized as well as some unusual properties of proteases’ pharmacokinetics and safety. Evidence of absorption as intact and biologically active molecules is provided and the concentration in the blood is shown to be sufficiently high to probably result in a variety of biological effects either by the different intrinsic proteolytic cleavage characteristics for proteins and certain surface receptors or their binding to specific (e.g. α₁-anti-trypsin, α₁-antichymotrypsin) or unspecified anti-proteases (α₂-macroglobulin).

**Gastrointestinal absorption of macromolecules**

Even today, the doubt on gastrointestinal absorption of larger peptides and proteins is often based on the view that the degradation of macromolecules like starch, fatty acids and proteins contained in nutrients is thought to be important for the absorption processes in the gastrointestinal tract. Several reasons are responsible for this thinking. First, pancreatic and intestinal proteases or peptidases produce free amino acids within the intestinal lumen. Their activity leads to the measurement of an increased amount of free amino acids in blood following ingestion of protein. Second, the characterization of amino acid carrier systems in the brush-border membrane of the small intestine further detracted attention from the possibility that forms other than amino acids enter the body during assimilation of a protein. Third, there were substantial methodological difficulties in quantifying the amounts of intact peptides and proteins crossing the gastrointestinal tract under unequivocally physiological conditions. These difficulties also included ensuring of an adequate specificity of analytical methods, overcoming the lack of methods for estimation, clearing by tissue or hydrolysis before and during sampling and handling [11-13].

**Absorption of peptides and proteins**

Early investigations on absorption of high molecular weight proteins were usually performed by oral application of radio-labelled proteins in animals. This method does not alter the enzymatic activity of the proteases, and the purification of labelled material for use by enteral tubes in sedated animals is easily performed. Only radioactivity was measured, and as shown by Skogh [14], the absorption rates estimated were too high. This is caused by splitting off iodine from the protein molecule by intestinal deiodinases or dissociation by changes in pH conditions. These results were confirmed by Bohe et al. [15] for human cationic trypsin provided into the duodenum of nine healthy individuals pre-treated with “cold”, i.e. non-radioactive iodine. The recovered radioactivity in plasma, characterized by dialysis and gel-filtration, was found to be in the form of free iodine. Furthermore, it was shown that pre-treatment with “cold” iodine prevented isotope binding to circulating plasma proteins, and, after intra-gastric application, the radioactive markers had been eliminated, especially in probes containing enzymes, had been adsorbed anywhere (e.g. thyroid gland), or had been bound to blood proteins.

The latter aspect holds true for the proteolytic enzymes discussed, as their free forms are physiologically bound to either specific (α₁-antichymotrypsin, α₁-anti-trypsin) or unspecified (α₂-macroglobulin) anti-proteases [16, 10]. This is a physiological necessity, as free, uncontrolled proteases, displaying their intrinsic proteolytic activity for prolonged times, may destroy biological material in body fluids after absorption. As shown later, this proteolytic activity may also be used to prove and quantify absorption across the gastrointestinal border into either blood or lymph.

With the development of new experimental techniques in addition to radio-labelling, the evidence for absorption of intact proteins across the gastrointestinal barrier increased. The new approaches were: (i) the detection of immunoreactive or both enzymatic and immunologic active specific proteins in body fluids; (ii) detection of antibodies against specific dietary proteins after ingestion in body fluids; (iii) animal experiments in vitro to follow the transport by techniques as specific as enzyme-linked immunosorbent assay and with establishment of the size by gel-filtration chromatography; and (iv) cytochemical and, preferably, immunocytochemical visualization in vitro of macromolecules after luminal administration, preferably in vivo. The latter two approaches have also provided evidence defining routes and mechanisms of transport emphasizing the use of endocytic trans-cellular processes. While each of these approaches has quite severe limitations,
the fact that all approaches point to the same conclusion (i.e. transport of small, but potentially biologically significant amounts of biologically active macromolecules) greatly strengthens the validity of this conclusion [13].

Immunological investigations confirming the absorption of not degraded dietary proteins in healthy humans include those on human β-lactoglobulin by Jakobsson et al. [17], on ovalbumin by Husby et al. [18], on bovine serum albumin, beta-lactoglobulin and ovalbumin by Paganelli and Levinsky [19], and on horseradish peroxidase [20]. However, the majority of experimental studies on macromolecule transport have been undertaken on animal intestine. Bockmann and Winbom [21] visualized by electron microscopy intracellular vesicles containing absorbed ferritin in hamster intestine and Walker and Isselbacher [22] these containing horseradish peroxidase.

Studies on absorption of proteins across biopsies of human intestine, although generally from children or from initially abnormal (e.g. malnourished) intestine, have provided generally similar information (e.g. [20, 23] and other references in [12]). Therefore, there is no reason to suspect major intra-species differences in the gastrointestinal handling of intact proteins.

In animal experiments, generally in rats, similar developments in knowledge have occurred concerning absorption of intact (or incompletely digested) peptides: as for large protein molecules, it was firmly believed that peptides are wholly hydrolyzed before absorption. This dogma has also had to be revised to account for transport of small but significant amounts of intact, biologically active peptides. The mechanisms for their trans-intestinal transport have now been characterized (e.g. [11, 12]) as described below. For example, in rats, biologically effective amounts of luteinizing hormone releasing hormone can be absorbed [24, 25], in animals and man vasopressin and analogues [26-30], and responses to oral thyreotropin-releasing hormone have been recorded in man [31].

Within the last four decades the view on the absorption of high molecular weight molecules (e.g. proteins and peptides) across the gastrointestinal barrier has completely changed. It is now accepted beyond reasonable doubt that significant (albeit small) amounts of macromolecules can be absorbed in intact and biologically active form [13]. Further, the fact that actual mechanisms of transport have been defined without controversy adds further weight to this conclusion.

Research of the last 30 years led to the schematic view of absorption models provided in Figure 1. Proteins are digested by multiple hydrolases, associated with membranes of columnar epithelial cells and secreted into the gut lumen. Specific carrier molecules transport amino acids, peptides and small proteins. In contrast, proteins are incorporated by M-cells present in the follicle-associated envelope of Peyer’s patches or through endocytosis by columnar epithelial cells. The incorporation of proteins by diffusion through intercellular spaces may be supported by intrinsic proteolytic activity of the administered proteases to solve the tight junctions or by reorganization processes of the epithelial layer (persorption). Detailed information on morphological and functional aspects of gastrointestinal absorption is outside the scope of this review and is described elsewhere.

Absorption of proteases

In line with the ongoing efforts to characterize the absorption of macromolecules, the first results were also published on absorption of proteases. As described, early investigations on absorption of proteases were also performed by oral application of radio-labelled material in animals with the same implications concerning the measured amounts. Another method used was the quantitative determination in plasma by measuring proteases’ intrinsic esterase activity on specific substrates e.g. N-benzoyl-L-arginine ethyl ester (BAEE) as substrate for trypsin and N-acetyl L-tyrosine ethyl ester (ATEE) as substrate for chymotrypsin, as well as haemoglobin for total proteolytic activity [32-34].

Evidence has accumulated that intestinal absorption is governed by several factors more important than molecular size. While many small molecules are not absorbed (for example, succinyl sulfathiazole, cathartics of the isatin group), ferritin with a molecular weight of 500,000 seems to be absorbed [35]. Martin and co-workers described absorption of [131]labeled trypsin from the buccal cavity and the intestine of experimental animals [36, 37], and
Miller [38] reported similar studies in man. Bogner and colleagues obtained comparable results with $^{131}$I-labelled trypsin in rats and $^{131}$I-labeled chymotrypsin in man [39]. Kabacoff and co-workers found ATEE activity in the blood of rabbits after intestinal or rectal administration of chymotrypsin [40]. Using the same method, Avakian demonstrated oral chymotrypsin absorption in man [41]. The same conclusion was drawn by Kabacoff (quoted by Bodi [42] on the basis of comparative oral and parenteral toxicity studies in mice. In 1964, Megel et al. [43] developed a sensitive method for detecting trypsin-like activity in rat plasma. For the first time they were able to determine that a minimal effective dose of 500 mg/kg of orally fed trypsin significantly increased plasma trypsin in rats [43].

First investigations with isolated rat intestines started in 1972, when Faudemay et al. [44] studied the transport of trypsin across the intestinal wall, using pieces of isolated rat jejunum and ileum. The intestine was filled with trypsin solution and was incubated in buffer. Aliquots of the acceptor buffer (serosal fluid) were taken and trypsin activity was determined by enzymatic reaction. Trypsin was found in the external medium in amounts increasing with time.

Detailed studies concerning the oral bioavailability of chymotrypsin were carried out by Moriya et al. in 1967 [45]. Radio-iodinated chymotrypsin was administered into rat intestine and the tissue distribution was determined. As a control, $^{131}$I-labeled potassium iodide was used, resulting in a different radioactive distribution. In addition, protein-bound radioactivity was proven as well as the stability of chymotrypsin after incubation with intestinal juices and increased serum esterase activity.

Absorption of the plant hydrolase bromelain (Ananas comosus) has been shown by Miller and Opher in 1964 [46]. They demonstrated an increased ability of the blood serum to digest casein after oral administration of enteric coated bromelain tablets. The bioavailability of bromelain has been studied in detail by White et al. in 1988 [47]. The total plasma radioactivity, TCA-precipitable $^{125}$I-compounds and the molecular weight profile of $^{125}$I-proteins, was measured after oral administration in rats. A maximum level, equivalent to 270 ng/ml bromelain, was found at 1 h after administration. Approxim
Review on absorption of serine and cysteine proteases

approximately 40% of the I\textsuperscript{125} in plasma could be precipitated by trichloroacetic acid. Electrophoresis analysis showed one major peak of radioactivity in the plasma samples, with a molecular weight of 26-32,000 Dalton. This is identical to the main molecular weight fraction in the bromelain mixture and corresponds to the molecular weight of the purified enzyme. In the 1 hour plasma sample this peak contained 0.003 per cent of the administered dose per millilitre.

In contrast, Seifert [48], administered radio-labeled I\textsuperscript{125}-bromelain intra-duodenal and took blood und lymph aliquots over a period of six hours, determined a bromelain absorption of about 50% of the administered dose after 6 hours (Figure 2). The samples were separated by radio-chromatography into high and low molecular weight labelled protein (Figure 3). About 80% of the radioactivity was found in high molecular weight fraction and its integrity was confirmed using Ouchterlony’s agar-double diffusion technique.

The calculated total amount of absorbed bromelain resulted in about 40% of the applied dose. The distribution of radioactivity in the different organs of the rat is shown in Table 2. Except the high radioactivity in kidney and thyroid gland it remains low with about 1% of the applied dose per gram organ weight. In the kidney 0.4% and in thyroid gland even 3.6% of the originally applied dose was determined per gram organ weight.

Further investigations on proteases used iodide\textsuperscript{125} radio-labelled material [49]. After purification, the proteases amylase, trypsin, chymotrypsin, papain, and pancreatin were administered by enteric tubes to rats while sedated. At the time points indicated in Figure 4, blood samples were taken and radioactivity was measured over 6 hours. It was confirmed by radio-chromatography and immunological methods that the proteases are qualitatively absorbed in the high molecular form. As shown, an increase in blood radioactivity was found in each animal. The findings are shown as percentages of the administered dose for the purpose of standardization. Pancreatin was absorbed most readily, followed by trypsin and chymotrypsin. Amylase was also

Figure 2. Radioactivity (percentage of the administered dose) in serum of rats after oral administration of radio-labelled enzymes (according to [50]).

Figure 3. Radio-chromatography with blood and lymph of rats after intraduodenal application of radio-labelled bromelain. Fraction I corresponds to high molecular weight bromelain and fraction II to low molecular fragments (B) (cpm = counts per minute; ml = millilitre) (according to [50]).
Review on absorption of serine and cysteine proteases

easily absorbed, but the absorption of papain was poorer. Measurements of radioactivity by lymph drainage up to six hours after administration led to equivalent amounts in the lymph for some enzymes and somewhat higher for others (data not shown).

The radio-labelled material in the blood and lymph samples was precipitable after addition of the specific antibodies to the proteases by forming the corresponding antigen-antibody complexes (double diffusion test in agar gel) [49]. High molecular weight material was also found after separation by Sephadex G20 gel filtration. The amount of high molecular weight material corresponds to 77% of amylase, 37% of chymotrypsin, 50% of pancreatin, 24% of papain and 54% of trypsin with reference to serum. The absorption rate of high molecular weight proteases from the originally applied material was calculated to be as about 45% for amylase, 14-21% for chymotrypsin, 18-20% for pancreatin, 6-15% for papain and 26-34% for trypsin as shown in Table 3.

However, it must be realized that in this case the radioactive portion of the radio-labeled molecules could be broken off during the process of absorption. The measured radioactivity may be quantitatively incorrect.

The presence of high molecular weight, obviously full size immune reactive bromelain, was confirmed in human blood samples after oral application [51, 52]. Plasma samples of the volunteers were drawn at the time of the maximum concentration after oral application and were immune precipitated with specific antibodies. The immune complexes were affinity isolated with Sepharose-bound goat IgG anti-rabbit IgG and dissociated with sodium dodecyl sulfate (SDS) and dithiothreitol (DTT). After separation of the plasma proteins by SDS gel electrophoresis, the protein bands were blotted from the gel to a PVDF-membrane. It was successively incubated with biotinylated anti-bromelain antibodies and extravidin-bound alkaline phosphatase. The color reaction catalyzed by the avidin-bound phosphatase revealed a protein band located at a molecular weight of 24 kDa (Figure 5, lanes 3-5). This value corresponds to the full-size bromelain molecule (lane 6). In the plasma of control individuals without bromelain application this protein band is not observed (lane 2).

Until this point, we had concerned ourselves with the immunological intactness and the di-

Table 2. Radioactivity in different compartments I125-labeled bromelain in rats of the intestine, absorption rate and distribution in different organs 6 hours after intra-duodenal application of 7.6 mg bromelain [48].

<table>
<thead>
<tr>
<th>Organ</th>
<th>% applied Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small intestine</td>
<td>49.6 ± 4.25</td>
</tr>
<tr>
<td>Large intestine</td>
<td>0.84 ± 0.26</td>
</tr>
<tr>
<td>Rate of absorption</td>
<td>49.6 ± 4.4</td>
</tr>
<tr>
<td>Lung</td>
<td>0.14 ± 0.025</td>
</tr>
<tr>
<td>Liver</td>
<td>0.10 ± 0.015</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.11 ± 0.017</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.42 ± 0.244</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.10 ± 0.018</td>
</tr>
<tr>
<td>Skin</td>
<td>0.16 ± 0.032</td>
</tr>
<tr>
<td>Thyroid gland</td>
<td>3.62 ± 0.561</td>
</tr>
</tbody>
</table>

(Mean of n = 10; ± standard deviation)

Figure 4. Radioactivity (percentage of the administered dose/gram blood) in serum of rats after oral administration of I123 radio-labeled proteases [50].
mensions of the molecules. The next question to be answered was whether proteases may retain their original activity after absorption, and whether this activity could be measured. Activity of amylase could not be found in the serum, but it could be detected by its activity in the lymph of the test animals [52]. As the activity of horse radish peroxidase has also been detected in serum [13], these two examples provide sufficient evidence that proteins may be absorbed as physiologically intact molecules.

It is expected that the amount of free proteases is small in body fluids due to their binding to specific or unspecific anti-proteases. To prove this binding, radioactive (\(^{125}\)I) bromelain was incubated with human citrated blood. A sample containing radioactive bromelain was subjected to high performance gel permeation chromatography. Individual fractions were examined by fuse rocket immune-electrophoresis (Figure 6B).

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|c|}
\hline
Protease & Applied amount of protease (mg) & Total rate of absorption (%) & Total rate of absorption (mg) & High molecular part (%) & Low molecular part (mg) & High molecular part (%) \\
\hline
Amylase & 10 & 59 & 5.9 & 77.1 & 73.7 & 43.5 - 45.5 \\
Chymotrypsin & 5 & 38.1 & 1.91 & 37.3 & 57.3** & 0.71 - 1.09 \\
Pancreatin & 2.25 & 36.6 & 0.82 & 50.3 & 55.7 & 0.41 - 0.46 \\
Papain & 5 & 26 & 1.3 & 24.1 & 60.6** & 0.31 - 0.79 \\
Trypsin & 5 & 49.6 & 2.48 & 54.2 & 68.7** & 1.34 - 1.7 \\
\hline
\end{tabular}
\caption{Quantitative analysis of absorption of proteases from rat intestine into serum and lymph [49].}
\end{table}

*Total rate of absorption was calculated as radioactivity applied by oral administration reduced by the part of residual activity of the intestine without differentiation into high or low molecular weight material; **Due to differences between concentration of high molecular weight proteases in serum and lymph, the calculated total amount of absorbed high molecular material is lower.

\textbf{Figure 5.} Presence of full-size bromelain in human plasma after oral intake of enteric-coated tablets in healthy male volunteers (figure 12 in [51]).

\textbf{Figure 6.} Binding of bromelain to plasma proteins. (A) Photographic film of radioactivity associated with plasma proteins. (B) Analysis of different fractions of separated plasma proteins with antibodies against \(\alpha_2\)-macroglobulin, \(\alpha_1\)-anti-trypsin and \(\alpha_1\)-anti-chymotrypsin (figure 14 in [51]).
zymes are absorbed as intact, high molecular weight molecules, retaining their activity as either free proteases or anti-protease bound complexes.

**Pharmacokinetics of proteolytic enzymes**

For pharmacokinetic investigations it was necessary to develop a test system to identify the intact, active protease molecules in the plasma of healthy volunteers after oral application [51, 52]. It was supposed, due to earlier experience, that the amount of molecules to be estimated in plasma is low. Therefore, sufficient amounts of proteolytic enzymes had to be administered orally and a low level of detection was necessary. Bromelain was chosen to start pharmacokinetic characterization of orally applied proteolytic enzymes in humans. The first strategy using the purified 2 major fractions of the original purchased bromelain product to raise antibodies in rabbits by an evaluated immunization cocktail and schedules of administration of the antigen failed due to the fact that the sensitivity (0.2 ng/well) was too low to allow for detection of small amounts of bromelain in the plasma of volunteers after oral application.

The next method developed used a non competitive one-species antibody ELISA. Two rabbit antibodies were raised against different bromelain epitopes and one of them was biotinylated. The first antibody was absorbed to the surface of the well, incubated with plasma probes containing bromelain and then incubated with the second biotinylated antibody. This method reached a limit of detection of about 100 pg/ml and a range of linearity of 20-2,000 pg/assay. No interference with plasma proteins was detected and the accuracy of inter-assay variation ranged from 95% to 103%. This antibody was used to confirm full-size bromelain in plasma (Figure 5).

The best method to solve the problem of determination of low levels of free proteases in serum from volunteers was the measurement of the intrinsic proteolytic activity of bromelain [53] by an antibody-capture method. Bromelain was first enriched by using the antibody to concentrate the enzyme from the plasma of volunteers and a specific, sensitive fluorogenic substrate was selected. Plasma samples were incubated first in wells previously coated with antibodies. This allowed bromelain to be captured by plate-bound antibody, facilitating the subsequent proteolytic activity measurement. A specific fluorogenic substrate, Z-Arg-coumarin, was used. The enzymatic hydrolysis of this non-fluorescent compound results in formation of a highly fluorescent coumarin. By combination of the two methods, a practical limit of detection of 1 ng bromelain/ml plasma could be reached.

A randomized, controlled, double-blind pharmacokinetic clinical trial has been performed in 19 healthy white males, aged 18-45 years. Fifteen volunteers received enteric-coated film tablets, each one containing 200 mg Bromelain. Four were administered placebo tablets. During the first day of the clinical trial, 3 tablets were administered at 0800 (time zero of the investigation), 1100, 1400, 1700, and 2000, followed by 5 tablets at 2300. The same pattern was followed on the second day. On the third day, only 3 tablets were administered at 0800. The volunteers received standard meals at 0900, 1200, 1530, and 1830. Blood samples (12 ml) were drawn each time before bromelain administration. Plasma was assayed for the presence of immunoreactive bromelain.

The subjects in the clinical trial showed significant variability in their plasma concentration profiles as well as in the profiles of individual proteolytic activity which matched the bromelain concentration profile (data not shown) and the median of immunoreactive bromelain shown in Figure 7. Consequently, the pharmacokinetic parameters peak plasma concentration (C_max), timing of peak plasma concentration (T_max), half-life (t_1/2), and area under the curve (AUC) were calculated independently for each experiment (Table 4), assuming a mono-compartmental model of distribution. For most volunteers, C_max was reached at ~48 hours with an average of 5 ng/ml. To estimate the t_1/2 of intestinally absorbed bromelain, the data of the decay phase of the second peak of the plasma concentration curve was used. Results from all volunteers show quite similar t_1/2 values of bromelain in blood (average value, 6.07 hours). The AUC for the period of 3-51 hours was calculated from the available experimental data as 82.2 ng/h/ml. The average blood concentration in this period, in individuals administered the protein orally, was 10.28 μg. The identity of bromelain was confirmed.

Using the new technique, a proteolytic enzyme combination consisting of trypsin, bromelain and rutosid was investigated in 20 healthy vol-
Review on absorption of serine and cysteine proteases

Volunteers using two different doses (4 and 8 tablets four times a day) for 4 days in a crossover design. Plasma concentration curves and those for hydrolytic activity of trypsin and bromelain are shown in Figure 8 [54-56].

As a result, a dose-dependent linear increase of the maximum plasma levels was detected in the plasma of the volunteers (Table 5) [57] and the course of the proteolytic serum activity paralleled that of the plasma concentration (Figure 8A and 8B). This means that active bromelain and trypsin were detectable in significant (physiological) albeit small concentrations as free proteases. Additionally, the information for papain is presented in Table 5.

To summarize, data from pharmacokinetic investigations show a dose-depending linearity of maximum plasma levels, despite a high inter-individual variability. The detected concentrations are comparable to the concentration of body’s own proteases, and the administration of protease-combinations does not interfere. The kinetic shows an unusual invasion and elimination kinetic (slow velocity of absorption, fast and 100% protein binding to anti-proteases) [57].

Mechanism of absorption of proteases

To further investigate the absorption mechanisms of proteases through the natural barrier of the gastrointestinal surface, the ex vivo, native tissue model using the human colon carcinoma cell line Caco-2 was used. The cells of a post-confluent culture of a Caco-2 mono-layer are similar to normal small intestinal enterocytes [58] and may be used for the measurement of selectivity as well as confluence and permeability by measuring the trans-epithelial electrical resistance (TEER) [59]. Transport of fluorescent markers normally not transported across the intact mono-layers is used as a

Table 4. Pharmacokinetic constants measured in plasma of healthy male volunteers after oral administration of enteric-coated tablets containing Bromelain [51], [52].

<table>
<thead>
<tr>
<th></th>
<th>C_{max} (ng/ml)</th>
<th>T_{max} (h)</th>
<th>t_{1/2} (h)</th>
<th>AUC (ng/h x ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volunteer 13 (Minimum)</td>
<td>1.83</td>
<td>51</td>
<td>6.2</td>
<td>31.2</td>
</tr>
<tr>
<td>Volunteer 08 (Maximum)</td>
<td>9.47</td>
<td>24</td>
<td>7.1</td>
<td>215.1</td>
</tr>
<tr>
<td>Mean</td>
<td>4.93</td>
<td>44</td>
<td>6.1</td>
<td>82.2</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>2.40</td>
<td>11</td>
<td>1.2</td>
<td>42.0</td>
</tr>
</tbody>
</table>

Table 5. Linear dose dependency of maximum plasma levels depending on different daily doses in pharmacokinetic investigations.

<table>
<thead>
<tr>
<th>No.</th>
<th>Bromelain</th>
<th>Trypsin</th>
<th>Papain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>daily doses [g]</td>
<td>max. plasma concentration [ng/ml]</td>
<td>daily doses [g]</td>
</tr>
<tr>
<td>1</td>
<td>1.08</td>
<td>2.5</td>
<td>0.38</td>
</tr>
<tr>
<td>2</td>
<td>1.44</td>
<td>3.8</td>
<td>0.72</td>
</tr>
<tr>
<td>3</td>
<td>2.16</td>
<td>4.8</td>
<td>0.77</td>
</tr>
<tr>
<td>4</td>
<td>2.88</td>
<td>5.6</td>
<td>1.44</td>
</tr>
</tbody>
</table>
Review on absorption of serine and cysteine proteases

Figure 8. Course of mean plasma concentration and hydrolytic activity of trypsin (A) and bromelain (B) in healthy volunteers after oral application of 2 dosages of an enzyme-rutoside combination product over 4 days (n =20) [54-56].

Figure 9. Influence of different proteases on Caco-2 monolayer integrity [modified according to Kolac et al., 1996 in reference 60]. (A) Influence of proteases on the Trans-Epithelial Electrical Resistance (TEER) of confluent Caco-2 mono-layer (○) control with fluorescein; (●) 17.75 F.I.P.-units/well trypsin in KRB; (▲) 1.23 F.I.P.-units/well bromelain in KRB; (■) 1.75 F.I.P.-units/well papain in KRB; (♦) 745 F.I.P.-units/well chymotrypsin in KRB. (B) Influence on transport of the transport marker substrate fluorescein (legend as before). FITC = Fluoroisothiocyanate; DMEM = Dulbecco’s Modified Eagle Medium; kDa = Kilo-Dalton; KRB = Krebs Ringer buffer.

The effects of proteases on the confluence and permeability of the differentiated Caco-2 monolayer were examined using fluorescein (MW 400) as a transport marker and by measuring the TEER [60]. A time and concentration dependent effect of trypsin, chymotrypsin, papain and bromelain both on TEER (Figure 9A) and on the transport of fluorescein across the monolayer was observed (Figure 9B). Proteases showed different effect sizes with the plant proteases being more active than the animal ones: (papain > bromelain > trypsin > chymotrypsin). Both effects, decrease of TEER simultaneously with the increase of marker transport, are indicative for a loosening of the tight junction between epithelial monolayer of Caco-2 cells. Further experiments confirmed that even fluorescent transport markers with a molecular weight of up to 600,000 Dalton are transported across the Caco-2 monolayer, and reversibility experiments revealed that the barrier function of the Caco-2 cell mono-layer was altered after incubation with proteases, but cells completely recovered after a few days. It seemed that some of the cells were removed from the monolayer, whereas cell death, caused by the enzymes, was unlikely.

The Caco-2 cell model is lacking some physiological factors with influence on the absorption of enzymes.
of macromolecules, e.g. the unstirred water layer, the presence of mucus, food, and components of blood plasma (albumin, antiproteases). The addition of mucin and albumin to the Caco-2 mono-layer had different effects on the activity of proteases on TEER and fluorescein transport, suggesting different mechanisms of action of proteases. The activity of trypsin and chymotrypsin was widely unaffected, that of papain was decreased by mucin and that of bromelain was increased by albumin. Albumin did not have any inhibitory effect on the proteases.

The absorption of proteases across the intestinal mucosa could be interpreted as self-enhanced paracellular diffusion through locally widened intercellular junctions. This hypothesis is further supported by the already known strong mucolytic activity of bromelain, trypsin and papain, based upon cleavage of amino acid binding sequence of mucus glycoproteins. Proteases are widely used in cell isolation for their ability to degrade extra-cellular matrix components. As shown above, the tightness of intracellular junctions is affected, opening the paracellular route across epithelium for non absorbable compounds. Within some tissues, papain has proved to be less damaging and more effective than other proteases. Enhancement of further low molecular weight compounds through the small intestine mucosa could also be shown [61].

Orally applied proteolytic enzymes reach concentration in the gastrointestinal tract several folds higher than in the used in vitro experiment, making the mechanism of paracellular transport even more reasonable, and the regeneration of the effects further support the safety of orally applied proteolytic enzyme in clinical use.

During the last decade, a possible role of proteases as signalling molecules has been emphasized with the discovery of a novel class of G-protein coupled receptors located on cell membranes that may be activated by proteolytic cleavage of their N-terminal extra cellular domain. Type 2 protease-activated receptors (PAR-2) are cleaved by serine-proteases such as trypsin and tryptase. PAR-2 is present in many intestinal cell types and particularly on epithelial cells. Multiple functions have been demonstrated in the gut for PAR-2, including epithelial permeability, mainly the intercellular permeability that is of paramount importance in the equilibrium between the external milieu (digestive contents) and the submucosal immune system. Alterations of both tissue and luminal levels of proteases or serine-protease activity may affect gut permeability and subsequently the immune status of the mucosa. Activation of PAR-2 on epithelial cells may directly affect cytoskeleton contraction by triggering phosphorylation of myosin light chain with subsequent changes in tight junction permeability. Thus, not only absorption of proteases is modified but also influences on changes in permeability occur. This opens a new field for further clinical evaluations [8, 9].

**Safety aspects for oral application of proteases**

As described, the single components of the orally applied proteolytic enzyme combination are absorbed in the gut, probably supported by a self-enhanced paracellular transport mechanism. They reach plasma concentrations of free proteases from customarily applied doses in the same order of magnitude of naturally occurring body's own proteases. The amount may be sufficient for their effects in the body.

The proteases are able to be detected in the serum after oral application in sub molecular concentrations, as it has been described for other physiologically active protease (e.g. proteases of the coagulation system). According to Klimmek [57] the maximum concentrations reached in plasma are a function of the daily dose of the single proteases and are independent from the combination of proteases applied (see Table 5). From a statistical point of view the correlation may be expressed by either \( y = a \cdot x + b \) or by geometric regression \( y = a \cdot x^b \). Initially, the linear regression is valid only for the dose intervals used. They only allow drawing a conclusion concerning the maximum concentration of free protease in the plasma reached in the investigated dose range. The amount of maximum concentrations below and above the investigated dose ranges may not be determined by a simple linear extrapolation due to the fact that the regression slopes are not going through zero. Despite that, maximum protease concentrations may be estimated by the help of the geometric regression for different daily doses of bromelain, trypsin and papain (Table 6). An increase of dose may, despite initial or continued dosing, not contribute to a main-
Review on absorption of serine and cysteine proteases

Table 6. Point estimates for the plasma concentrations \( y \) [ng/ml] of free soluble bromelain, trypsin and papain dependent from daily dose \( x \) [g] in humans deriving from corresponding geometric regression equation [57].

<table>
<thead>
<tr>
<th>Daily dose ( x ) [g]</th>
<th>Bromelain ( y = 2.5586x^{0.7863} )</th>
<th>Trypsin ( 2.7061x^{0.8466} )</th>
<th>Papain ( 1.6916x^{0.9867} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>0.41854</td>
<td>0.38525</td>
<td>0.17442</td>
</tr>
<tr>
<td>0.2</td>
<td>0.72183</td>
<td>0.69278</td>
<td>0.34564</td>
</tr>
<tr>
<td>0.3</td>
<td>0.99288</td>
<td>0.97651</td>
<td>0.51567</td>
</tr>
<tr>
<td>0.4</td>
<td>1.24491</td>
<td>1.24580</td>
<td>0.68494</td>
</tr>
<tr>
<td>0.5</td>
<td>1.48367</td>
<td>1.50485</td>
<td>0.85363</td>
</tr>
<tr>
<td>0.6</td>
<td>1.71237</td>
<td>1.75601</td>
<td>1.02188</td>
</tr>
<tr>
<td>0.8</td>
<td>2.14702</td>
<td>2.24027</td>
<td>1.35730</td>
</tr>
<tr>
<td>1.0</td>
<td>2.55880</td>
<td>2.70610</td>
<td>1.69160</td>
</tr>
<tr>
<td>1.5</td>
<td>3.51963</td>
<td>3.81437</td>
<td>2.52375</td>
</tr>
<tr>
<td>2.0</td>
<td>4.41302</td>
<td>4.86627</td>
<td>3.35215</td>
</tr>
<tr>
<td>3.0</td>
<td>6.07011</td>
<td>6.85922</td>
<td>5.00119</td>
</tr>
<tr>
<td>4.0</td>
<td>7.61090</td>
<td>8.75080</td>
<td>6.64279</td>
</tr>
</tbody>
</table>

Maintained increase of the concentration of free proteases in serum. Therefore, the risk of a strong increase of the concentration of free proteases is low.

The safety of orally applied protease combinations is also documented by calculation. The occurrence of free proteases in the blood is limited by time and derives from a rapid complex formation with anti-proteases (e.g. \( \alpha_2 \)-macroglobulin). The complex formation follows the law of mass action and reaches for the absorbed protease 100% with time. Not only the foreign protease but also body’s own proteases verifiably have only a tiny chance to escape the catching by \( \alpha_2 \)-macroglobulin.

The amount and the occurrence of \( \alpha_2 \)-macroglobulin have been excessively investigated without detecting a complete deficiency of this macroglobulin. It indicates that \( \alpha_2 \)-macroglobulin is of vital importance in man. Depending on the velocity, about 0.2% (being very rational from a practical point of view) and after ingestion of 800 mg protease about 16% (practically very improbable) of the naturally occurring \( \alpha_2 \)-macroglobulin reservoir might be irreversibly and cumulatively occupied and made available for a rapid elimination in the liver. A complete occupation of the body’s \( \alpha_2 \)-macroglobulin reserve by orally applied proteases is more than unlikely. Therefore, the components of the protease combinations are safe even in high doses due to these pharmacological mechanisms.

Biological activity of proteolytic enzymes

The biological activity of proteases is deriving either from their site-specific hydrolytic cleavage activity or their binding to anti-proteases.

The hydrolytic activity is essential in biological processes, and thousands of different proteases are already known. Their activity can be differentiated by their substrate specificity, pH-dependency, confirmation and modification (e.g. anti-protease binding). After oral administration and absorption, free proteases may be active in body fluids and even damaged tissue despite their low concentrations (pmol - nmol) as long as the complex with anti-proteases is not formed [13, 50, 62].

The binding to anti-proteases is required to protect the organism from self destruction. If proteases’ concentration, from whatever source, increases locally, their activity must be controlled. Mostly, proteases are activated as a response to changes in the environment e.g. metabolic changes, injury, invading pathogens or negative chronic effects like oxidative stress or inflammation, either acute or chronic by auto reactive antibodies. This makes them an ideal signal molecule to participate in regulatory processes. As all free proteases are harmful to cells, tissues and even organisms, they are all controlled by unique mechanisms such as inactive...
precursor generation or binding to anti-proteases. Obviously the organism does not differentiate proteases according to their origin (foreign plant or animal as well as body's own proteases). The anti-proteases can bind a variety of proteases [63, 7].

**Biological activity of anti-protease complexes**

It is not known which residual hydrolytic activity is expressed by anti-protease bound proteases. Low molecular weight substrates and probably peptides may be hydrolysed.

Figure 10 shows protease molecules entrapped by α2-macroglobulin [63]. The resulting complexes are recognized by low density lipoprotein receptor-related proteins on the surface of blood cells and hepatocytes for rapid elimination and activating the production of anti-proteases; a necessity, if their consumption due to acute or chronic processes is increased.

Anti-proteases have a half life of several days. Structural changes of the α2-macroglobulin molecules after binding of 1 or 2 protease molecules in both cases generate a “fast”-form, which is, in contrast to the “slow”-form, eliminated within minutes by macrophages and hepatocytes [64, 65].

As shown in Figure 11, there is a significant increase of plasma anti-protease levels after oral administration of an enzyme-rutoside combination (Wobenzym®) [50]. The advantages of such increases of hydrolytic activity and anti-protease levels need to be further investigated. Hydrolytic serum activity is reduced with increasing age and is under diurnal control. Several chronic (arthritis) [66] or acute diseases (heavy burns) are accompanied by reduced anti-protease levels (high consumption by free proteases) [67]. Beneficial effects from consumption of food additives or therapeutics might arise by increase of anti-protease levels after oral administration of protease combinations.

The activated anti-protease-complex with α2-macroglobulin has an essential function in the course of acute or chronic inflammation. It is able to bind different cytokines and growth factors. Obviously, this mechanism is essential for the elimination of such mediators from the inflammatory tissue [68-71].

The activated anti-protease-complex is subject to further modifications. Once formed, it may be oxidized depending on the local conditions (e.g. pro-inflammatory radical formation). The oxidized form preferentially binds cytokines that stimulate the immune process (TH-1-cytokines). If the oxidizing activity is reduced during ongoing inflammation/repair process, the preference is changed to preferential binding of TH-2-
cytokines and growth factors. This process might be a trigger mechanism in the course of the immune reaction [72, 73].

The central role of the activated anti-protease-complex is further substantiated by the knowledge that it induces G-protein-receptor-coupled signal transduction and second messenger production. A serpine-enzyme complex receptor [74, 75] as well as an activated α2-macroglobulin-signalling receptor different from lipoprotein-related protein receptor involved in degradation of the complex have been described [76].

**Biological activity based on hydrolytic cleavage**

In addition to the activities mediated by the complex with an anti-protease, orally provided free proteolytic enzymes may influence cell activity by different hydrolytic mechanisms. First, cells may be (in-)activated or desensitized by proteolytic cleavage of cell surface receptors generating soluble peptides or proteins. As an example the interaction of trypsin and chymotrypsin on protease activated receptors (PAR) is described [77]. Actually four different subtypes have been described with about 30% homology but less sequence homology. They owe diverse functions, are participating in many biological processes, and span across the membrane (7 loops) with an intracellular binding site for G-protein(s) [78]. The presently known biological effectors are shown in **Table 7**.

The different proteases cleave the PAR receptor at a particular site, unmasking a previously cryptic N-terminal sequence of the receptor, defined as “tethered ligand”. This tethered ligand sequence interacts with the conserved second extra cellular loop and activates the same receptor. If no tethered ligand is formed, but a respective protease is bound, the receptor is desensitized. If a tethered ligand is bound though no cleavage of the tethered ligand sequenices occurred, the receptor is inactivated.

Trypsin is an agonist of PAR-1 (weak) and PAR-2 (strong), whereas chymotrypsin is an antagonist by desensitizing the receptor's activity. Steinhoff et al. described biologic activity of trypsin and chymotrypsin on signal transduction cascades and production of second messengers leading to apoptosis, supporting host defense, regulating immune modulation, inflammation and others [78].

**Summary**

Orally administered proteolytic enzymes can be detected transiently as intact, high molecular weight, physiologically active protein molecules, either free (nanomolar concentrations) or in a complex with anti-proteases in plasma, lymph, or injured tissue. Data from pharmacokinetic investigations reveal a dose-depending linearity of maximum plasma levels, a high inter-individual variability, plasma concentrations comparable to body's own proteases, no interference by administration of protease-combinations, an unusual invasion and elimination kinetic (slow velocity of absorption, fast and 100% protein binding to anti-proteases). Oral application of proteases leads to increased proteolytic serum activity and increased plasma concentrations of the corresponding anti-proteases. Biological activity of orally administered proteases is determined by their proteolytic activity as free proteases on cell surface receptors (e.g. protease activated receptors) or soluble peptides/proteins and their activity in a complex formed with anti-proteases. The complex of proteolytic enzyme and anti-protease induces increased plasma concentrations of anti-proteases and elimination of anti-protease-complexes and cytokines.

Oral administration of enteric coated tablets containing proteolytic enzymes of plant and animal origin may stabilize or probably enhance a

**Table 7.** (De-)Activators and desensitizer of protease-activated-receptor (PAR) 1 to 4 (modified according to [78])

<table>
<thead>
<tr>
<th>Activators</th>
<th>Deactivators</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin (+++); [Trypsin, Granzyme A, Plasmin (-)]</td>
<td>Cathepsin G, Elastase, Plasmin, Proteinase 3, Chymotrypsin (Sensitivity, down regulation)</td>
</tr>
<tr>
<td>Trypsin (+++), Trypsate, Factor Xa, Proconvertin</td>
<td>Cathepsin G, Plasmin, Proteinase 3</td>
</tr>
<tr>
<td>Thrombin, Trypsin, Factor X</td>
<td>Cathepsin G, Elastase</td>
</tr>
<tr>
<td>Thrombin, Trypsin, Cathepsin G</td>
<td>?</td>
</tr>
</tbody>
</table>
variety of physiological and immunological processes even in healthy consumers.

Acknowledgements

Figures 5, 6, 10, 11 have been kindly authorized by Springer Science and Business Media and the authors. The author wishes to thank Sonja Raum, Munich, for critical reading of the manuscript.

Address correspondence to: Dr. Gerhard Lorkowski, GL Pharma Consulting Research & Development (GL Pharma CR&D), Hubertusstr. 77a, D-82131 Gauting, Germany; Tel: +49-89-803696; Fax: +49-89-803644; Mobile: +49-171-641 99 75; E-mail: gerhard.lorkowski@gmx.de

References

Review on absorption of serine and cysteine proteases


Review on absorption of serine and cysteine proteases


[58] Pinto M. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol Cell 1983; 47: 323-330.


Naturopathic Treatment of Rotator Cuff Tendinitis Among Canadian Postal Workers: A Randomized Controlled Trial

OREST SZCZURKO,1 KIERAN COOLEY,1 EDWARD J. MILLS,2 QI ZHOU,2 DAN PERRI,2 AND DUGALD SEELY1

Objective. To explore the effectiveness of naturopathic care (NC) on rotator cuff tendinitis using a prospective randomized clinical trial design.

Methods. Canadian postal workers with rotator cuff tendinitis for a duration of >6 weeks were randomized to receive NC (n = 43) or standardized physical exercises (PEs; n = 42) over 12 weeks. Participants in the NC group received dietary counseling, acupuncture, and Phlogenzym (2 tablets 3 times/day). The PE intervention group received passive, active-assisted, and active range of motion exercises and matched placebo. The primary outcome measure was the Shoulder Pain and Disability Index (SPADI), and secondary outcomes were the pain visual analog scale (VAS), Short Form 36 (SF-36), Measure Yourself Medical Outcomes Profile (MYMOP), and shoulder maximal range of motion. Participants and assessors were blinded to group and placebo allocation.

Results. Seventy-seven participants (87%) completed ≥8 weeks of the trial. Final total SPADI scores decreased by 54.5% (P < 0.0001) in the NC group and by 18% (P = 0.0241) in the PE group. Between-group differences in changes to SPADI scores showed statistically significant decreases in shoulder pain and disability in the NC group compared with the PE group (P < 0.0001). Significant differences between groups were also observed in the pain VAS, MYMOP, SF-36, and shoulder extension, flexion, and abduction, with the NC group showing superiority in each outcome. No serious adverse reactions were observed.

Conclusion. NC and PE provided significant improvements, with greater improvement in shoulder function in the NC group compared with the PE group. Statistically significant improvements in quality of life measures were observed in the NC group as compared with the PE group.

INTRODUCTION

Shoulder pain is the most common extraspinal symptom encountered in primary care clinics, and in clinical frequency is exceeded only by low back and neck pain (1). The incidence has been estimated at 11.2 per 1,000 person-years (2). In a number of workplace health studies, shoulder pain was second only to back pain in workers’ compensation insurance claims, chronicity, and work impact (3–5). It carries a high social cost by increasing worker absenteeism, decreasing employees’ quality of life and productivity, and contributing to a lower level of job satisfaction (6).

Many shoulder conditions are associated with dysfunction of the rotator cuff (7–9). Rotator cuff tendinitis is caused by inflammation of one of the supraspinatus, infraspinatus, subscapularis, and teres minor muscle tendons (10). Conventional treatments for rotator cuff tendinitis without complete tears include rest, muscle strengthening, pain management, physiotherapy, corticosteroid injections, and surgery (11,12). These therapies exhibit limited or inconsistent long-term success, and are often viewed by patients as a last resort (13,14). Alternative strategies range from physical therapies such as massage and manipulation to energy-based interventions such as acupuncture. Naturopathic medicine combines several approaches and is commonly used for this condition.
We sought to evaluate the potential for the combined efficacy of a naturopathic approach including acupuncture, dietary advice, and hydrolytic enzymes in the treatment of rotator cuff tendinitis.

PATIENTS AND METHODS

Recruitment. The study took place from March to June 2007, and involved Canadian postal employees who were members of the Canadian Union of Postal Workers. Information sheets were distributed throughout eligible Canadian postal sites. Interested employees received an information package that included an informed consent form, background information explaining the purpose of the study, a description of the care to be provided, a question and answer sheet regarding study participation, and contact information for study enrollment.

Participants were primarily from a central letter processing plant in Toronto, Ontario, Canada; however, a minority of the study population came from the surrounding Toronto area mail delivery depots. Participants included day, evening, and night shift letter and package sorters, as well as outdoor letter carriers and drivers.

Intervention. Naturopathic medicine is a system of medicine with a primary goal of identifying and treating the cause of illness (15). It is a regulated and licensed profession in 5 Canadian provinces and 15 states in the US. Naturopathic doctors practicing in the province of Ontario receive training in and utilize the following therapies: dietary interventions, herbal and nutritional supplements, physical therapy, counseling, acupuncture, and intravenous therapies after specialized training. This study evaluated the combined use of acupuncture, dietary changes, and the use of a supplement as a semistandardized naturopathic intervention for the treatment of rotator cuff tendinitis.

On its own, acupuncture has been shown to be effective in numerous musculoskeletal syndromes (16,17) and for shoulder pathologies, including rotator cuff tendinitis (18). Kleinhenz et al demonstrated that acupuncture treatment provided significant improvement over placebo on measures, including upper extremity pain, range of motion, strength, and impact on daily activities (19.2 versus 8.4 on the Constant-Murley score) (19).

An antiinflammatory diet may be beneficial in the treatment of rotator cuff tendinitis. Inflammation characterized by the production of inflammatory cytokines contributes to a range of acute and chronic human diseases (20,21). Omega-3 polyunsaturated fatty acids found in oily fish decrease the production of these inflammatory markers (22). Encouraging the consumption of fish high in n-3 polyunsaturated fatty acids may be beneficial to patients with rotator cuff tendinitis (23). Other dietary suggestions include the addition of soybeans and cherries, which can also have pain-decreasing effects, possibly due to their antiinflammatory properties (24). A diet high in plant flavonoids has also been shown to decrease inflammatory processes (25).

Along with acupuncture and an antiinflammatory diet, the naturopathic intervention also evaluated the safety and efficacy of Phlogenzym (Mucos Pharma, Puhonice, Czech Republic) in the treatment of rotator cuff tendinitis. Phlogenzym is composed of the hydrolytic enzymes bromelain and trypsin, and the bioflavonoid rutin. European pharmacologic and medical literature cites the mechanisms of action of oral hydrolytic enzymes as fibrinolytic, antiedematous, antiinflammatory, and analgesic (26–30). Hydrolytic enzymes activate macrophages and natural killer cells by breaking down immune complexes interleukin-1β and interleukin-6 (31). Rutin has been shown to normalize pathologically increased vascular permeability (32). Phlogenzym also contributes to the degradation of plasma proteins that invade the interstitial space during acute inflammation. Following injury, Phlogenzym has been shown to decrease fibrin deposits (fibrinolysis) and restore microcirculation (32).

Trypsin has antioxidant properties that can result in a decrease of the inflammatory response to injury (32). Both bromelain and trypsin can reduce platelet aggregation, lymphocyte adherence, and decrease activation of protease-activated receptor 2, causing vasodilation and prevention of platelet, leukocyte, and erythrocyte adherence (32). Bromelain also exhibits fibrinolytic activities in dissolving fibrin clots (32). Combined, these effects have been shown to decrease pain, reduce inflammation and swelling, and be as effective as the nonsteroidal antiinflammatory drug (NSAID) diclofenac in several musculoskeletal symptoms (27–29,33).

Recruitment and initial visit. All potential participants were scheduled for a 1-hour assessment with 1 of the 2 trial investigators, both of whom are naturopathic doctors. During this initial visit, any outstanding questions were answered, an informed consent form was signed and witnessed, and the intake interview and physical examination were conducted to evaluate participant eligibility.

Physical measurements assessed during the initial visit included height, weight, body mass index, sitting left arm blood pressure, shoulder range of motion, and orthopedic tests. The range of motion tests included flexion, extension, abduction, adduction, internal rotation, and external rotation of both the affected and unaffected shoulder. Ranges were measured using a goniometer/inclinometer (Universal Inclinometer U101; Performance Attainment Associates, Lindstrom, MN) and performed by a second coordinator blinded to the patient’s history. Orthopedic tests included the Neer Impingement, Speeds, Apprehension, and Subscapularis Lift tests, and were also performed by the intake physician and blinded coordinator. The diagnosis of rotator cuff tendinitis was confirmed by the blinded coordinator.

The intake interview included family history, patient history, review of systems, and assessment for inclusion and exclusion criteria. Participants also received a diary sheet to document their diet, medication, and concurrent therapy use at home. All women of reproductive age were screened for pregnancy via a human chorionic gonadotropin urine test. At the end of the initial visit, potential participants completed the Shoulder Pain and Disability
Effectiveness of Naturopathic Care on Rotator Cuff Tendinitis

Index (SPADI), the Short Form 36 (SF-36), and the Perceived Benefit Questionnaire.

To be considered for inclusion, participants must have been between 18 and 65 years old, judged as able to adhere to the given protocol, had pain in at least 1 shoulder for the previous 6 weeks or more, and had symptoms consistent with rotator cuff tendinitis.

Participants were excluded if they could not comply with the study protocol, had no shoulder pain or range of motion limitations consistent with rotator cuff tendinitis at the time of assessment, had previously identified allergies to any ingredients of Phlogenzym, were receiving corticosteroid injection therapy, were taking daily warfarin or antibiotics, abused substances such as alcohol or illegal drugs, had a severe concurrent illness, or were pregnant or breastfeeding. The use of periodic or ad libitum pain medications was monitored, but was not a reason for exclusion.

Two licensed naturopathic doctors provided onsite delivery of care (OS, KC). This study was a pragmatic randomized controlled trial comparing naturopathic medicine with standard first-line physical exercise treatment for rotator cuff tendinitis. The treatment interventions were planned and carried out for 12 weeks. Upon determination of eligibility and collection of baseline information, participants were randomized using age- and sex-matched computer randomization to either naturopathic care (NC; active group) or physical exercise (PE; control group). Allocation concealment using central randomization was preserved up to the point of treatment and was maintained by the blinded coordinator performing range of motion and orthopedic test assessment. Although the analyst and participants were blinded to allocation and supplements were delivered using identical-looking tablets for all supplements and placebo, it was not possible to mask the interventions from the participants or the clinicians delivering care. Care was taken in the informed consent to ensure that participant blinding was preserved and to keep expectation biases equal within both treatment groups.

Treatment groups. Naturopathic care. Participants receiving NC were seen once per week for 30 minutes for a total of 12 weeks to receive specific treatment for rotator cuff tendinitis. Dietary counseling specific to the individual patient was given, with special emphasis on reducing alcohol consumption and increasing consumption of fish, berries, fruits, vegetables, nuts, and whole grains. Standardized acupuncture treatments were performed at each visit, with needle insertion at LI15, SI14, SI19, SI10–13, and BL41–46, plus up to 4 Ashi points of pain. Acupuncture needles were inserted and briefly stimulated using a perpendicular thrusting technique until the patient reported a dull aching sensation. All needles were left in for a duration of at least 10 minutes, with at least one instance of restimulation using the perpendicular thrusting technique according to the Gunn method (34). Restimulation was done to address the attenuation of sensation and response that can accompany needle insertion. The supplement Phlogenzym containing 90 mg of bromelain, 48 mg of trypsin, and 100 mg of rutin was approved for the study by the Natural Health Products Directorate of Health Canada and provided to participants with instructions to take 2 tablets 3 times per day for the 12-week duration of the trial. Supplements were dispensed to participants every 4 weeks, and compliance was assessed through pill count every 4 weeks for the duration of the trial. Pills were dispensed in blister packs marked only with the study code, subject number, dose instructions, and contact information of the investigators.

Physical exercise. Participants randomized to the control group were seen once weekly for 30 minutes for a total of 12 weeks. Participants received PEs following a protocol shown to be effective for addressing pain consistent with work place–related rotator cuff tendinitis (35). During the treatment visits, participants received a series of passive, active-assisted, and active range of motion muscle strengthening and joint therapy consistent with standard physiotherapy for shoulder injuries (36). Hands-on shoulder muscle and joint therapy was designed to increase shoulder range of motion and assist in recovery and therapy for repetitive strain injuries. Placebo tablets were matched to the Phlogenzym for color, smell, and taste, and consisted of an inert fiber substance. Placebo capsules were dispensed in the same number, container, and schedule as the Phlogenzym given to the NC group.

The control group did not receive dietary counseling, acupuncture, or Phlogenzym, but did receive identical frequency matched supplement placebo, PEs, and hands-on shoulder muscle and joint therapy. Treatment duration and frequency were equal in both groups. Careful consideration was given toward the design and implementation of treatments to provide an effective treatment for rotator cuff tendinitis while creating an adequately matched placebo to the active group. As such, the control group treatment regimen contained elements similar to the NC group consisting of an individualized treatment approach, a well-developed therapeutic doctor-patient relationship, patient motivation, and the consumption of a pill.

Primary outcome. Administered at baseline and at 4, 8, and 12 weeks, the SPADI was the primary outcome measure. It is a validated self-report questionnaire measuring the pain and disability associated with shoulder pathology (37). The SPADI consists of 13 items; participants rate how their shoulder function is affected by 2 subscales: pain (5 items) and disability (8 items). Scores can range from 0 to 50 on the pain scale, from 0 to 80 on the disability scale, and from 0 to 130 on the total scale. An increasing score indicates increasing pain or disability.

Secondary outcomes. Secondary outcomes were performed at baseline and at 4, 8, and 12 weeks.

The SF-36 is a self-administered, 36-item questionnaire that measures health-related quality of life in 8 domains. Each domain is scored separately from 0 (worst score) to 100 (best score). Two summary scores can be calculated from the information obtained in the 8 domains: the physical function and mental health summary scores (38).

The pain visual analog scale (VAS) is a self-adminis-
tered single-item questionnaire that asks the participant to assess their average degree of shoulder pain experienced over the last week. Scores range from 0 to 7, where 0 = no pain at all and 7 = severe pain.

The Measure Yourself Medical Outcomes Profile (MYMOP) is a patient-centered outcome questionnaire with internal consistency and construct validity (39,40). It is used in primary care settings as a means of garnering and quantifying qualitative patient experiences. At baseline, patients choose 2 personally relevant symptoms of greatest importance to their health and rate these symptoms on a 7-point VAS. The same 2 symptoms are then reevaluated at each data-gathering followup visit. Ranging from 0 to 6, higher scores correspond to a lower satisfaction (i.e., worse) level of health.

The flexion, extension, abduction, adduction, internal rotation, and external rotation of the affected shoulder were assessed by a coordinator blinded to treatment and using a goniometer/inclinometer. Participants were instructed to actively move the shoulder in the required direction as far as possible.

Statistical analysis. All analyses were performed by a statistician (QZ) under blinded conditions using SAS/STAT, version 9.1 (SAS Institute, Cary, NC). Baseline visit and week 12 visit data were summarized by the mean ± SD. When missing data occurred at week 12, its value was replaced by the value at week 8 as part of the intent-to-treat analysis. For each group, the treatment effect at week 12 was compared with that at baseline and assessed by the paired t-test. The mean difference and its 95% confidence interval and P value were reported. The independent t-test was used to compare the difference in treatment effect between the active (NC) and control (PE) groups. The mean differences of the treatment effects, 95% confidence intervals, and P values are reported below.

To determine a 20% reduction in SPADI scores, and assuming a population SD of 20%, a sample size of 37 in each treatment arm was calculated, providing 80% power and a 5% alpha level (41). A commitment to enroll at least 80 participants was made to supplement expected loss to followup. All patients with available data were analyzed according to the arm to which they were randomized. We summarized the baseline data and the data at week 12 by the mean ± SD. The means over the 12-week period were plotted separately for the outcomes of the SPADI, SF-36, VAS, and range of motion. To assess the treatment effect for each group, we calculated the mean change in scores between groups at week 12 and baseline. We also calculated the mean changes between groups to examine the group effect. The statistical significance of the changes for each group was tested using the paired t-test; the exact 2-sided P value is reported. The 2 sample t-tests were performed to compare the change scores between groups.

The outcomes reported from these analyses include total, pain, and disability SPADI scores; pain VAS, MYMOP, and SF-36; and range of motion.

RESULTS

Recruitment and followup. Recruitment led to the screening of 103 participants during initial visits. Fourteen of those screened did not qualify: 1 was unknowingly pregnant, and others did not have shoulder pain or had torn shoulder tendons and therefore did not meet inclusion criteria. In total, 89 patients were randomized and enrolled in the study. Four of the 89 patients decided to not start the study after reconsideration or became unreachable before the first treatment visit. None of these 4 participants withdrew with the knowledge of what type of treatment they would be receiving. Of the 85 participants who started treatment, 17 (10 control, 7 active) did not complete the 12-week course of study: 1 participant broke her leg, 6 became unreachable, and 10 could not commit the time or lost interest. Of the 43 participants who started treatment in the NC group, 41 completed week 8 and 36 completed week 12. Of the 42 participants who started treatment in the PE group, 36 completed week 8 and 32 completed week 12.

Baseline characteristics. Randomization was stratified based on age and sex. Both treatment groups had similar characteristics, including baseline SPADI score, range of motion, age, and sex (Table 1).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Naturopathic care (n = 43)</th>
<th>Physical exercise (n = 42)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>50.7 ± 8.16</td>
<td>50.9 ± 7.86</td>
<td>0.98</td>
</tr>
<tr>
<td>Sex, no. (%)</td>
<td>18 (42)</td>
<td>17 (40)</td>
<td>0.85</td>
</tr>
<tr>
<td>Men</td>
<td>25 (58)</td>
<td>25 (60)</td>
<td></td>
</tr>
<tr>
<td>Total SPADI score</td>
<td>77.64 ± 29.38</td>
<td>69.61 ± 24.11</td>
<td>0.83</td>
</tr>
<tr>
<td>Flexion range of motion†</td>
<td>122.15 ± 35.91</td>
<td>124.78 ± 37.09</td>
<td>0.96</td>
</tr>
<tr>
<td>Extension range of motion†</td>
<td>36.29 ± 11.71</td>
<td>39.03 ± 11.97</td>
<td>0.87</td>
</tr>
<tr>
<td>Abduction range of motion†</td>
<td>101.17 ± 44.24</td>
<td>104.47 ± 44.73</td>
<td>0.96</td>
</tr>
<tr>
<td>Adduction range of motion†</td>
<td>35.76 ± 11.00</td>
<td>35.93 ± 12.00</td>
<td>0.99</td>
</tr>
</tbody>
</table>

* Values are the mean ± SD unless otherwise indicated. SPADI = Shoulder Pain and Disability Index.
† Maximum active range of motion.
Study treatments. The treatments were well received, as demonstrated by both the pain VAS and good compliance rates in both groups. The mean total of missed supplements was 5.5 pills per week (86% compliance) in the NC group and 7.4 pills (81% compliance) in the PE group throughout the 12-week duration of the trial.

Outcomes. Significant improvements from baseline on the primary outcome measure (total SPADI score) were seen in both the NC (−42.34; P < 0.001) and PE (−12.68; P = 0.024) groups (Table 2 and Figure 1). Total SPADI scores improved significantly more in the NC group as opposed to the PE group (−29.7; P < 0.0001). The NC group also showed statistically significant differences in SPADI pain and disability subscales. The SPADI pain subscale decreased by −18.70 (P < 0.0001) in the NC group and by −5.7 (P = 0.0094) in the PE group. The SPADI disability subscale demonstrated this trend with a 21.64 decrease (P < 0.0001) in the NC group versus a 6.00 decrease (P = 0.081) in the PE treatment group.

The pain VAS showed significant reductions in pain (P < 0.001) in the NC group (Table 3 and Figure 2). The improvements reported on the SPADI and VAS questionnaires are supported by range of motion improvements in measured maximal flexion (P < 0.0001), extension (P < 0.0001), and abduction (P < 0.0001) of the affected shoulder in the NC group as compared with the PE group. Maximal adduction was found to have a nonsignificant trend toward improvement between groups (P = 0.568) (Table 4).

The MYMOP patient-centered outcome measure was used to allow the patient to evaluate the progression of the 2 health concerns most significant to them. The first symptom showed significant improvement in both the NC (−2.2; P < 0.0001) and PE (−1.29; P < 0.0001) groups. The second symptom significantly improved in the NC group (−2.52; P < 0.0001), whereas the PE group did not achieve significance (−0.66; P = 0.0443).

The SF-36 showed statistically significant differences (P < 0.01) between the NC and PE groups in all subcategories except social functioning, which showed a trend toward improvement (P = 0.038). The NC group showed the greatest improvement over the PE group in role physical (P = 0.0015), bodily pain (P = 0.0004), and interestingly in role emotional (P = 0.0020).

Adverse events. Two adverse reactions were observed in the NC group: 1 patient had loose stool for one day and another reported mild sedation for one day. The PE group reported 1 incident of mild abdominal discomfort, 1 participant reported diarrhea, 1 reported flatulence, 1 reported constipation, and 1 patient briefly experienced moderate skin flushing, burning ears, and a mild tingling sensation. Gastrointestinal upset was most commonly reported in both groups. All reported reactions resolved within 1 or 2 days, and were considered to be mild by the participants reporting them.

DISCUSSION

The results of this clinical trial should be of interest to patients and clinicians alike. This study showed a reduction in rotator cuff tendinitis with the use of standardized physical exercises routinely used by physical therapists. These results are consistent with previous reports of the efficacy of physical exercises and therapies in the treatment of shoulder pain (42). In comparison, naturopathic treatment showed a clinically and statistically significant
improvement in shoulder pain and quality of life measures as compared with the control standard.

Antinflammatory whole-food diets emphasizing omega-3 fatty acids, plant flavonoids, green tea, and cruciferous and yellow vegetables have been shown to reduce inflammation (22,23,43). It has previously been shown that the avoidance of known proinflammatory irritants such as alcohol, smoking, soft drinks, refined grains, and processed meats will lower inflammation (43,44).

Phlogenzym has demonstrated antinflammatory effects in previous research (26–32,45,46). Trypsin can result in a decrease in the inflammatory response to injury. Combined with bromelain, it can reduce platelet aggregation, lymphocyte adherence, and decrease protease-activated receptor 2 activation. Phlogenzym has been shown to decrease pain, reduce inflammation and swelling, and be as effective as the NSAID diclofenac in several musculoskeletal symptoms.
This study.

A factorial design would allow the characterization of the treatment packages. Future research trials employing a multi-component therapies of either the NC or PE as such it is impossible to ascertain the precise effect of the individual components, including dietary changes, acupuncture, and Phlogenzym use or visits to a naturopathic doctor occurred. The lack of a no treatment control group limits the external validity of this study; however, given the complex and holistic approach to the care employed, it would be impossible to separate the contextual effects implicit in the interventions used.

Naturopathic treatment including dietary advice, acupuncture, and ingestion of a proteolytic enzyme appears to be safe and effective in providing significant benefit over standard therapy in the treatment of chronic rotator cuff tendinitis in the Canadian postal worker population. Standard therapy including physical therapy and strengthening/stretching exercises was shown to also achieve improvements under the same conditions but not to the same degree as the semistandardized naturopathic treatment approach. Future research is required to examine the effects of the individual components of treatments.

Maintaining internal and external validity in pragmatic randomized controlled trials is a challenge (47,48). Our study design attempted to maximize external validity by having few exclusion criteria and including some flexibility or variability in study interventions based on individual needs (e.g., specific advice given in dietary counseling). However, one specific enzymatic supplement was used to treat all study participants in the NC group despite the range of potential antiinflammatory natural health products (49). Treatment decisions made by the naturopathic doctors in the study were limited as a result, thus decreasing the external validity of our study.

Internal validity was achieved by attempting to account for nonspecific effects of treatments in both groups and absence of assessment bias, and by preventing contamination (47). Both the NC and PE groups received therapeutic doctor-patient relationships, patient motivation and education, a physical therapy, and a pill. Assessment and data analysis were conducted by assessors blinded to group allocation. No contamination of the PE group with respect to Phlogenzym use or visits to a naturopathic doctor occurred. The lack of a no treatment control group limits the internal validity of this study; however, given the complex and holistic approach to the care employed, it would be impossible to separate the contextual effects implicit in the interventions used.

Table 4. Maximal range of motion goniometer readings before and after NC and PE treatment*

<table>
<thead>
<tr>
<th>Outcome†</th>
<th>Baseline, mean ± SD</th>
<th>Week 12, mean ± SD‡</th>
<th>Change from baseline</th>
<th>95% CI</th>
<th>P</th>
<th>Mean difference between groups</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flexion</td>
<td>NC 122.15 ± 35.91</td>
<td>159.39 ± 25.97</td>
<td>37.24</td>
<td>28.66, 45.83</td>
<td>&lt; 0.0001</td>
<td>40.94</td>
<td>28.58, 53.3</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>PE 124.78 ± 37.09</td>
<td>121.08 ± 40.53</td>
<td>-3.69</td>
<td>-12.88, 5.49</td>
<td>0.4196</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>NC 36.29 ± 11.71</td>
<td>42.39 ± 11.18</td>
<td>6.10</td>
<td>2.80, 9.40</td>
<td>0.0006</td>
<td>9.68</td>
<td>4.90, 14.46</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>PE 39.03 ± 11.97</td>
<td>35.44 ± 10.26</td>
<td>-3.58</td>
<td>-7.16, -0.1</td>
<td>0.0495</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abduction</td>
<td>NC 101.17 ± 44.24</td>
<td>148.63 ± 34.73</td>
<td>47.46</td>
<td>37.98, 56.94</td>
<td>&lt; 0.0001</td>
<td>46.57</td>
<td>31.21, 61.94</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>PE 104.47 ± 44.73</td>
<td>105.36 ± 45.05</td>
<td>0.89</td>
<td>-11.83, 13.61</td>
<td>0.8880</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adduction</td>
<td>NC 35.76 ± 11.00</td>
<td>35.39 ± 7.42</td>
<td>-0.37</td>
<td>-2.67, 1.94</td>
<td>0.7499</td>
<td>-0.81</td>
<td>-3.63, 2.01</td>
<td>0.5683</td>
</tr>
<tr>
<td></td>
<td>PE 35.83 ± 12.60</td>
<td>36.28 ± 11.05</td>
<td>0.44</td>
<td>-1.11, 2.00</td>
<td>0.5664</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* NC = naturopathic care; PE = physical exercise; 95% CI = 95% confidence interval.
† Maximum active range of motion of the affected shoulder was assessed with a goniometer by a coordinator blinded to treatment. The NC group achieved significant improvements over the PE group in flexion, extension, and abduction.
‡ As part of the intent-to-treat analysis, the week 8 value was used for week 12 if the value was missing.
ACKNOWLEDGMENTS

We thank The Canadian Union of Postal Workers and the Canada Post Corporation, Joint Benefits Committee for providing access to the study population, and Chris Mazzuchin, BSc, PT, ND, for his physical therapy expertise and advice in the design of this study.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Seely had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Szczurko, Cooley, Mills, Seely.

Acquisition of data. Szczurko, Cooley, Zhou.


REFERENCES


VITAMINS AND MINERALS

Effect of vitamin D3 supplementation in black and in white children: A randomized, placebo-controlled trial.
J Clin Endocrinol Metab. 2016.

A randomized trial of nutrient supplements to minimise psychiatric illness after a natural disaster.
Psych Res. 2015.

Oral high-dose multivitamins and minerals after myocardial infarction.

Hip bone loss is attenuated with 1000 IU but not 400 IU daily vitamin D3: a 1 year double-blind RCT in postmenopausal women.
J Bone Min Res. 2013.

Effect of selenium supplementation on CD4+ T-cell recovery, viral suppression and morbidity of HIV-infected patients in Rwanda: a randomized controlled trial.
AIDS. 2015.

Effect of selenium supplementation on CD4 T-cell recovery, viral suppression, morbidity and quality of life of HIV-infected patients in Rwanda: study protocol for a randomized controlled trial.
Trials. 2011.
Effect of Vitamin D₃ Supplementation in Black and in White Children: A Randomized, Placebo-Controlled Trial

Kumaravel Rajakumar, Charity G. Moore, Jonathan Yabes, Flora Olabopo, Mary Ann Haralam, Diane Comer, Jaimee Bogusz, Anita Nucci, Susan Sereika, Jacqueline Dunbar-Jacob, Michael F. Holick, and Susan L. Greenspan

Department of Pediatrics (K.R., F.O., M.A.H.), and Center for Research on Health Care (C.G.M., J.Y., D.C.), University of Pittsburgh, Pittsburgh, Pennsylvania 15213; Department of Medicine (J.B., M.F.H.), Boston University School of Medicine, Boston, Massachusetts, 02118; Department of Nutrition (A.N.), Georgia State University, Atlanta, Georgia 30302; University of Pittsburgh School of Nursing (S.S., J.D.-J.), Pittsburgh, Pennsylvania 15213; and Department of Medicine (C.G.M., J.Y., D.C., S.L.G.), University of Pittsburgh, Pittsburgh 15213

Context: Dosages of vitamin D necessary to prevent or treat vitamin D deficiency in children remain to be clarified.

Objective: To determine the effects of vitamin D₃ 1000 IU/d on serum 25-hydroxyvitamin D [25(OH)D], PTH, and markers of bone turnover (osteocalcin and collagen type 1 cross-linked C-telopeptide) in black children and white children, and to explore whether there is a threshold level of 25(OH)D associated with maximal suppression of serum PTH concentration.

Design: Healthy 8- to 14-year-old Pittsburgh-area black (n = 84) and white (n = 73) children not receiving vitamin supplements, enrolled from October through March from 2008 through 2011, were randomized to vitamin D₃ 1000 IU or placebo daily for 6 months.

Results: The mean baseline concentration of 25(OH)D was 20 ng/mL in both the vitamin D-supplemented group and the placebo group (19.8 ± 7.6 and 18.8 ± 6.9 ng/mL, respectively). The mean concentration was higher in the supplemented group than in the placebo group at 2 months (26.4 ± 8.1 vs 18.9 ± 8.1 ng/mL; P = .0001) and also at 6 months (26.7 ± 7.6 vs 22.4 ± 7.3; P = .003), after adjusting for baseline 25(OH)D, race, gender, pubertal status, dietary vitamin D intake, body mass index, and sunlight exposure. Increases were only significant in black children, when examined by race. The association between 25(OH)D and PTH concentrations was inverse and linear, without evidence of a plateau. Overall, vitamin D supplementation had no effect on PTH and bone turnover.

Conclusions: Vitamin D₃ supplementation with 1000 IU/d in children with mean baseline 25(OH)D concentration 20 ng/mL effectively raised their mean 25(OH)D concentration but failed to reach 30 ng/mL. Vitamin D supplementation had no effect on PTH concentrations. (J Clin Endocrinol Metab 100: 3183–3192, 2015)

Maintaining adequate vitamin D status is essential for calcium homeostasis and skeletal health. However, hypovitaminosis D is common in healthy children living in the northeastern United States, and its prevalence and severity are greater in black than in white children (1). Circulating concentration of 25-hydroxyvitamin D [25(OH)D] is the recognized biomarker of vitamin D status. Definition of a 25(OH)D cutoff level for optimal skel-

Abbreviations: BMI, body mass index; CTx, collagen type 1 cross-linked C-telopeptide; CV, coefficient of variation; DBP, vitamin D-binding-protein; OC, osteocalcin; 25(OH)D, 25-hydroxyvitamin D; Δ, change.
et al health lacks consensus. The Institute of Medicine (IOM) recommends concentrations of 25(OH)D ≥20 ng/mL as optimal for skeletal health and defines vitamin D deficiency as a concentration <12 ng/mL and vitamin D insufficiency as concentrations 12 to 20 ng/mL (2). The IOM estimates that concentrations of ≥16 ng/mL and ≥20 ng/mL would be adequate for ensuring the skeletal health needs of 50 and 97.5%, respectively, of US children. The Endocrine Society and other experts in the field have suggested a target level ≥30 ng/mL for optimal skeletal health (3, 4).

Concentrations of 25(OH)D associated with maximal suppression of serum PTH concentration, indicated by a plateauing of the PTH concentration, have been used for defining vitamin D sufficiency in adults. Such data in children are limited and varied. Hill et al (5) found no inflection point in the inverse association between 25(OH)D and PTH in a cross-sectional study of 7- to 18-year-old children. Although Maguire et al (6) demonstrated plateauing of PTH at a 25(OH)D concentration of 42.8 ng/mL in a cross-sectional study of 1- to 6-year-old children, vitamin D supplementation (0, 400, 1000, 2000, and 4000 IU/d for 12 wk) in 9- to 13-year-old children had no effect on PTH concentrations despite dose-dependent increases in 25(OH)D concentrations (7).

Confounding effects of sun exposure and other determinants of vitamin D photoproduction pose challenges for estimating the amount of dietary vitamin D needed to achieve and maintain a defined 25(OH)D level (2). Those considerations notwithstanding, the IOM’s dietary reference intakes for vitamin D were calculated without regard for variations in skin color, race, or sunlight exposure. Delineating the racial differences in response to vitamin D supplementation and exploring levels of 25(OH)D associated with vitamin D sufficiency is relevant for formulating dietary guidelines. In addition, data regarding the effect of vitamin D supplementation on bone turnover are limited. Therefore, we initiated a pharmacological challenge with 1000 IU of vitamin D₃ daily (five times the prevailing adequate intake for vitamin D) (8) in black children and white children to examine the responsive changes in 25(OH)D, PTH, and bone turnover. The objective of this study was to determine the effects of supplementation with 1000 IU of vitamin D₃ on serum concentrations of 25(OH)D, PTH, and markers of bone turnover in black children and white children. We utilized the longitudinal design of our study to determine whether there was a threshold level of 25(OH)D associated with maximal suppression of serum PTH concentration.

## Subjects and Methods

### Study design and participants

We enrolled healthy 8- to 14-year-old children from Pittsburgh and Kittanning, Pennsylvania (latitude/longitude: 40.4° N/80° W and 40.8° N/79.5° W, respectively) in a randomized, double-blind, placebo-controlled trial of vitamin D₃ 1000 IU daily (ClinicalTrials.gov identifier: NCT00732758). The children were enrolled October through March of 2008 through 2011. Children receiving vitamin preparations underwent a 1-month washout before enrollment.

Subjects were recruited from the Primary Care Center of the Children’s Hospital of Pittsburgh of the University of Pittsburgh Medical Center (UPMC) and the Children’s Community Pediatrics-Armstrong practice in Kittanning, and through advertisements posted in the offices of the Children’s Hospital-affiliated Pediatric PittNet, a practice-based pediatric research network. Study procedures subsequent to enrollment were conducted either at the UPMC Montefiore Clinical and Translational Research Center or at the Children’s Community Pediatrics-Armstrong practice. The study was approved by the University of Pittsburgh Institutional Review Board. Signed informed parental consent and subjects’ assent were obtained before research participation. The race of subjects was specified by their parents.

### Randomization and intervention

Randomization was stratified by race using a 1:1 allocation ratio and a permuted block scheme with block size of 4. Children received either vitamin D₃ 1000 IU or placebo in a single tablet once daily. The allocation scheme was generated by a study statistician using R version 2.7.0. The vitamin D₃ and placebo tablets were manufactured by Douglas Laboratories, were similar in color, and were dispensed in identical containers labeled either A or B. A sealed envelope system was used for the assignments. Children, parents, and the investigative team were blinded to the treatment assignment until the study was completed. Study medications were made in two batches. The average vitamin D₃ content of the vitamin D₃ tablet, measured as previously described (9), in the first batch around the midpoint of its shelf-life was approximately 1129 IU; in the second batch at the end of the trial, it was approximately 1140 IU.

### Compliance

Compliance was assessed by pill count at the 2- and 6-month follow-up visits and was validated in a subset of 90 subjects by an electronic medication event monitoring system (MEMs 6 Track Cap; AARDEX).

### Study measurements

#### Anthropometry, sun exposure, skin color, dietary intake, and pubertal status

We measured height and weight and calculated body mass index (BMI) at study entry and at the 6-month follow-up visit. At study entry and exit, we assessed: summertime sunlight exposure characteristics; melanin index from the forehead, back of the hand, and inner arm using a handheld dermospectrophotometer (DSM II Colormeter; Cortex Technology); and dietary intake of vitamin D and calcium using a validated Youth and Adolescent Food Frequency Questionnaire (10, 11). We estimated
Tanner stage by physical examination (12, 13) and ascertained the parent-reported Fitzpatrick sunreactive skin type (14–16) at study entry.

Biochemical assessments

We collected blood samples by venipuncture in a nonfasting state throughout the day. We measured serum calcium, phosphorus, albumin, 25(OH)D, PTH, osteocalcin (OC; marker of bone formation), and collagen type 1 cross-linked C-telopeptide (CTx; a marker of bone resorption) concentrations at baseline and at 2- and 6-month follow-up visits. Calcium, phosphorus, and albumin concentrations were measured at the UPMC Clinical Chemistry laboratory. Serum 25(OH)D, PTH, OC, and CTx concentrations were measured at the Vitamin D, Skin, and Bone Research Laboratory at Boston University Medical Center. Serum 25(OH)D concentrations were measured using liquid chromatography-tandem mass spectrometry (17). The intra-assay and interassay coefficients of variation (CVs) were 6% and 10%, respectively. The 25(OH)D assay is Center for Disease Control certified, and National Institutes of Standards and Technology standards were used for confirmation of the standard curves. Serum 3-epi-25(OH)D is excluded in the reported levels. Serum PTH concentrations were assayed using a Human Bioactive PTH 1–84 Elisa kit (Immuno landslide, Inc); intra-assay and interassay CVs were 7 and 9%, respectively. Serum OC concentrations were measured by Micro Vue OC enzyme immunoassay kit (Quidel); intra-assay and interassay CVs were 5 and 10%, respectively. Serum CTx concentrations were measured by Serum CrossLaps ELISA kit (Immunodiagnostics Systems); intra-assay and interassay CVs were 1.8–3% and 8.0–10.9%, respectively.

Statistical analysis

We based parameter estimates for sample-size analyses on our earlier study of changes in PTH concentration in response to vitamin D3 supplementation (18). Assuming that the concentration of 25(OH)D would reach >20 ng/mL in the supplemented group and would remain ≤20 ng/mL in the placebo group and with two-sided α = 0.05, we estimated that 160 children would be needed to detect effect sizes in serum 25(OH)D and PTH concentrations of approximately 0.5, with power of 81 to 88%. Assuming that 50% of the children were black and 50% white, we had 79% power to detect a correlation of at least –0.3 between PTH and 25(OH)D concentrations within each race group (α = 0.05). We anticipated an attrition rate of 5% and planned to enroll a total of 168 children.

We used intention-to-treat analyses when testing the effects of vitamin D supplementation relative to placebo. We tested for treatment group or racial differences in categorical measures using χ² or Fisher’s exact tests and in continuous measures using t tests. Concentrations of 25(OH)D, the primary study outcome, did not require transformation. We compared mean 25(OH)D concentrations using analysis of covariance, controlling for baseline 25(OH)D, race, gender, pubertal status, BMI, dietary intake of vitamin D, and sunlight exposure.

To explore threshold effects of 25(OH)D on PTH concentrations, we conducted analyses similar to those described by Hill et al (5) and Maguire et al (6) using linear and cubic spline regressions. Scatterplots were constructed superimposed with both fitted lines and Lowess (locally weighted scatterplot smoothing) curves to visually examine nonlinearity. We used mixed-effects modeling to examine the associations between PTH and 25(OH)D concentrations. This allowed us to account for repeated measures within children across time and adjust for the baseline covariates of BMI, race, and calcium intake and for the time-varying covariates of 25(OH)D and season. In the models, season was based on the date of visit and categorized as fall/winter/early- to midspring (October through May), and late spring/summer (June through September). We assessed cross-sectional between-child associations using baseline measures and within-child associations using changes from baseline.

Results

Of 304 eligible children, 157 were enrolled (Figure 1), 141 in Pittsburgh and 16 in Kittanning. Eighty-four of the children were black and 73 were white. The vitamin D3-supplemented and placebo groups were similar at baseline (Table 1). Baseline characteristics between vitamin D and placebo groups, when examined within each race group, were also balanced except for hand melanin index in black children (Table 1). However, hand melanin index measurements were missing in 12 black children.

Effects of Vitamin D₃ supplementation

Effects on mean 25(OH)D concentrations

Baseline 25(OH)D concentrations were not different between the vitamin D₃-supplemented and placebo groups, and both were <20 ng/mL (Table 2). At the 2- and
Table 1. Baseline Characteristics of Enrolled Children

<table>
<thead>
<tr>
<th></th>
<th>All Children</th>
<th>Black Children</th>
<th>White Children</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vitamin D</td>
<td>Placebo</td>
<td>Vitamin D</td>
</tr>
<tr>
<td></td>
<td>Group</td>
<td>Group</td>
<td>Group</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>P Value</td>
<td>n</td>
</tr>
<tr>
<td>Demographic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>78</td>
<td>.07</td>
<td>42</td>
</tr>
<tr>
<td>Black</td>
<td>42</td>
<td></td>
<td>23 (54.8)</td>
</tr>
<tr>
<td>Hispanic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>African</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Hispanic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Under 12</td>
<td>1.1 ± 2.0</td>
<td>.48</td>
<td>1.1 ± 1.9</td>
</tr>
<tr>
<td>Weight, kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summer</td>
<td>48.4 ± 18.0</td>
<td>.80</td>
<td>49.7 ± 18.2</td>
</tr>
<tr>
<td>Height, cm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal weight (BMI &lt;85th %tile)</td>
<td>44.5 (56.4)</td>
<td>.72</td>
<td>20 (47.6)</td>
</tr>
<tr>
<td>Overweight (BMI ≥95th %tile)</td>
<td>14 (17.9)</td>
<td></td>
<td>8 (19.1)</td>
</tr>
<tr>
<td>Tanner stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>30 (38.5)</td>
<td>.99</td>
<td>14 (33.3)</td>
</tr>
<tr>
<td>II</td>
<td>20 (25.6)</td>
<td>.09</td>
<td>12 (28.6)</td>
</tr>
<tr>
<td>III</td>
<td>12 (15.4)</td>
<td>.52</td>
<td>6 (14.3)</td>
</tr>
<tr>
<td>Obese (BMI ≥95th %tile)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malanin index*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I (easy burn, no tan)</td>
<td>4 (5.3)</td>
<td>.26</td>
<td>0 (0)</td>
</tr>
<tr>
<td>II (easy burn, slight tan)</td>
<td>14 (18.4)</td>
<td></td>
<td>0 (0)</td>
</tr>
<tr>
<td>III (burn, then tan)</td>
<td>15 (19.7)</td>
<td>.52</td>
<td>4 (9.8)</td>
</tr>
<tr>
<td>IV (no burn, good tan)</td>
<td>36 (47.4)</td>
<td>.76</td>
<td>30 (73.2)</td>
</tr>
<tr>
<td>V (never burn, marked tan)</td>
<td>7 (9.2)</td>
<td></td>
<td>7 (17.1)</td>
</tr>
<tr>
<td>Melanin index*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forehead</td>
<td>51.9 ± 18.2</td>
<td>.40</td>
<td>66.9 ± 13.5</td>
</tr>
<tr>
<td>Hand</td>
<td>54.2 ± 18.1</td>
<td>.28</td>
<td>69.0 ± 13.5</td>
</tr>
<tr>
<td>Sunscreen use, yes</td>
<td>51.2 ± 18.6</td>
<td>.52</td>
<td>64.6 ± 13.7</td>
</tr>
<tr>
<td>Sunscreen use frequency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Often</td>
<td>63 (85.1)</td>
<td>.32</td>
<td>33 (84.6)</td>
</tr>
<tr>
<td>Sometimes</td>
<td>28 (37.8)</td>
<td>.27</td>
<td>6 (15.4)</td>
</tr>
<tr>
<td>Laboratorial data</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium, mg/dl</td>
<td>9.7 ± 0.4</td>
<td>.43</td>
<td>9.8 ± 0.38</td>
</tr>
<tr>
<td>Phosphorus, mg/dl</td>
<td>5.0 ± 0.5</td>
<td>.14</td>
<td>4.9 ± 0.49</td>
</tr>
<tr>
<td>Albumin, g/dl</td>
<td>4.2 ± 0.3</td>
<td>.64</td>
<td>4.3 ± 0.33</td>
</tr>
<tr>
<td>25(OH)D, ng/ml</td>
<td>19.8 ± 7.6</td>
<td>.38</td>
<td>16.6 ± 7.0</td>
</tr>
<tr>
<td>PTH, pg/mL</td>
<td>36.9 ± 19.6</td>
<td>.83</td>
<td>43.4 ± 20.8</td>
</tr>
<tr>
<td>OC, mg/ml</td>
<td>94.2 ± 44.5</td>
<td>.18</td>
<td>88.0 ± 19.9</td>
</tr>
<tr>
<td>Dietary intake</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium, mg/d</td>
<td>1164.3 ± 602.2</td>
<td>.71</td>
<td>1157.0 ± 600.9</td>
</tr>
<tr>
<td>Vitamin D, μl/d</td>
<td>253.6 ± 141.0</td>
<td>.23</td>
<td>239.4 ± 132.1</td>
</tr>
<tr>
<td>Vitamin D-deficient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25(OH)D &lt; 20 ng/mL</td>
<td>42 (54)</td>
<td>.69</td>
<td>30 (71)</td>
</tr>
</tbody>
</table>

6-month follow-up visits, concentrations were higher in supplemented children, and in black supplemented children considered as a separate subgroup. The increase in 25(OH)D concentration in supplemented children was greater in black than in white children at 2 months but not at 6 months. Mean 25(OH)D concentrations were lower generally in black children than in white children. Analysis of covariance parameter estimates for the 25(OH)D differences at 2 and 6 months are shown in Supplemental Table 1.

**Children with baseline 25(OH)D concentrations <20 ng/mL**

Of this subgroup of children who received vitamin D₃ supplementation, concentrations at 2 months were between 20 and 30 ng/mL in 65% (24 of 37) and ≥30 ng/mL in 3% (one of 37). Corresponding proportions in the children who received placebo were 14% (five of 36) and 6% (two of 36), respectively. At 6 months, concentrations in supplemented children were between 20 and 30 ng/mL in 47% (17 of 36) and ≥30 ng/mL in 14% (five of 36). Cor-
responding proportions in the children who received placebo were 53% (18 of 34) and 0% (0 of 34), respectively. The proportions in black children and white children were similar.

**Magnitude of changes in 25(OH)D concentration in relation to baseline concentration**

Lower baseline 25(OH)D concentration was associated with greater change in 25(OH)D concentration at 2 months overall ($r = -0.32; P < .001$) and in black children ($r = -0.49; P < .001$), but not in white children ($r = -0.0005; P = 1.0$). The associations were similar for 6-month changes overall ($r = -0.44; P < .001$), and both in black children ($r = -0.58; P < .001$) and white children ($r = -0.24; P = .048$).

**Changes in 25(OH)D concentrations in relation to dietary intake of vitamin D and sun exposure**

Baseline dietary intake of vitamin D was not correlated with changes in 25(OH)D concentrations at 2 and 6 months. The change in 25(OH)D concentrations was higher in children with >2 hours vs ≤2 hours of sunlight exposure (2 months: $3.6 ± 8.2$ ng/mL vs $0.9 ± 6.0$ ng/mL, $P = .16$; 6 months: $5.3 ± 7.4$ vs $2.6 ± 4.6$ ng/mL, $P = .035$). This association varied by race and skin pigmentation at 2 months but not at 6 months (Supplemental Table 2). The direction of the association was the same and was significant among white children and in children classified as light-skinned (skin type I–III) but not in black children and dark-skinned children (skin type IV–V), respectively.

**Effects on PTH concentrations**

Vitamin D supplementation did not have an impact on mean PTH concentrations at 2 and 6 months (Table 2). At baseline, mean PTH concentrations were higher in black children than in white children in each of the two treatment groups and remained so throughout the duration of the study.

**25(OH)D-PTH associations**

In unadjusted analyses, both the between-children association and the within-child association were negative in all children (standardized coefficients, $-0.89 [P < .001]$ and $-0.24 [P = .03]$, respectively); the between-children association was stronger than the within-child association ($P < .001$). Calcium intake did not modify these associations. Adjusting for season, BMI, calcium intake, and race attenuated the strength of both associations (standardized coefficients, $-0.64 [P = .013]$ and $-0.14 [P = .23]$, respectively).

The 25(OH)D and PTH association, by race, was significant only in black children (between-children: black, standardized coefficient, $-0.92 [P < .001]$; white, $-0.38 [P = .10]$; within-child: black, $-0.30 [P = .038]$; white, $-0.13 [P = .39]$). In black children, the between-children association was stronger than the within-child association ($P = .011$). Calcium intake did not modify these associations. Adjusting for season, BMI, and calcium intake attenuated the strength of both associations (standardized coefficients, $-0.89 [P < .001]$ and $-0.24 [P = .13]$, respectively).

---

**Table 2.** 25(OH)D and PTH Concentrations by Treatment Group and Race

<table>
<thead>
<tr>
<th></th>
<th>Total Sample (n = 157)</th>
<th>Black (n = 84)</th>
<th>White (n = 73)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vitamin D Group</td>
<td>Placebo Group</td>
<td>P Value</td>
</tr>
<tr>
<td>Mean 25(OH)D concentrations, ng/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>$19.8 ± 7.6$ (78)</td>
<td>$18.8 ± 6.9$ (79)</td>
<td>.38</td>
</tr>
<tr>
<td>2-mo f/u</td>
<td>$24.6 ± 8.1 (72)$</td>
<td>$18.9 ± 8.1 (64)$</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>6-mo f/u</td>
<td>$26.7 ± 7.6 (70)$</td>
<td>$22.4 ± 7.3 (63)$</td>
<td>.003</td>
</tr>
<tr>
<td>2 mo – baseline Δ</td>
<td>$6.1 ± 7.6$</td>
<td>$0.17 ± 7.0$</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>6 mo – baseline Δ</td>
<td>$6.5 ± 7.4$</td>
<td>$3.4 ± 6.7$</td>
<td>.01</td>
</tr>
<tr>
<td>Mean PTH concentrations, pg/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>$36.9 ± 19.6 (78)$</td>
<td>$37.6 ± 19.0 (79)$</td>
<td>.83</td>
</tr>
<tr>
<td>2-mo f/u</td>
<td>$35.1 ± 17.6 (72)$</td>
<td>$32.7 ± 13.6 (64)$</td>
<td>.37</td>
</tr>
<tr>
<td>6-mo f/u</td>
<td>$35.5 ± 16.3 (69)$</td>
<td>$33.5 ± 17.0 (62)$</td>
<td>.51</td>
</tr>
<tr>
<td>2 mo – baseline Δ</td>
<td>$-1.8 ± 15.4$</td>
<td>$-4.7 ± 16.2$</td>
<td>.29</td>
</tr>
<tr>
<td>6 mo – baseline Δ</td>
<td>$-1.5 ± 13.9$</td>
<td>$-3.4 ± 20.0$</td>
<td>.52</td>
</tr>
</tbody>
</table>

Abbreviation: f/u, follow-up. Data are shown as mean ± SD (number).

**a** P values of 25(OH)D group differences at 2- and 6-month follow-up are adjusted for baseline 25(OH)D, race, BMI, diet vitamin D, gender, pubertal status, and sunlight exposure; **b** P values of PTH group differences are unadjusted; **c** P values of 25(OH)D Δ and PTH Δ group differences are unadjusted.

**b** $P < .0001$ for the difference between black children and white children in the corresponding treatment group.

**c** $P < .001$ for the difference between black children and white children in the corresponding treatment group.

**d** $P < .01$ for the difference between black children and white children in the corresponding treatment group.

**e** $P < .05$ for the difference between black children and white children in the corresponding treatment group.
We had limited power to detect a threshold concentration of 25(OH)D associated with maximal suppression of PTH. This was largely because few of the 25(OH)D data points were above 40 ng/mL relative to other studies that have explored this association (5, 6, 19). The Lowess curves (Figure 2, A–C) of the between-children associations in all children and in the race subgroups were linear, without evidence of plateauing. Visually suggestive plateauing of PTH concentrations around 30 ng/mL in black children and 40 ng/mL in white children were not significant for nonlinearity (P = .27 and P = .67, respectively).

**Effects on OC and CTx concentrations (Table 3)**

Overall, vitamin D₃ supplementation had no effect on mean concentrations of OC and CTx. Black children had lower mean concentration of OC and higher mean concentration of CTx than white children at baseline; and in the placebo group, their mean OC concentrations remained significantly lower than white children at 2 and 6 months.

The effect of treatment varied by race for changes in OC at 6 months (race-by-treatment interaction term, P < .001). In vitamin D-supplemented black children, mean OC concentration was significantly higher than the placebo group at 6 months. There was no effect on CTx in black children. In white children, however, mean OC and CTx concentrations were both lower in the vitamin D-supplemented group than the placebo group at 2 and 6 months.

**25(OH)D and bone turnover relationships (Table 4)**

At baseline, neither OC nor CTx was associated with 25(OH)D in all children, and in black children and white children when adjusted for intervention, pubertal status, sex, and race. At 2 and 6 months, change (Δ) in 25(OH)D was positively associated with the Δ OC overall and in white children when adjusted for intervention, pubertal status, race, sex, and baseline 25(OH)D.

**Compliance**

Three children, all in the placebo group, discontinued study medication; one was started on vitamin D supplementation by the treating physician, and the other two experienced unrelated events. By pill count, more than 80% of prescribed doses were taken by children in both the vitamin D and placebo groups. Proportions of prescribed doses taken did not differ between the two treatment arms and were confirmed by the validation procedure. Compliance did not vary by race.

**Adverse effects**

No child developed hypercalcemia.
Discussion

We have shown that vitamin D₃ supplementation of 1000 IU/d during fall and winter in children with baseline mean 25(OH)D concentrations <20 ng/mL was safe and effective to raise their mean concentrations of 25(OH)D to 20–30 ng/mL but not to ≥30 ng/mL. Of the supplemented children with baseline 25(OH)D concentrations <20 ng/mL, only 14% reached concentrations ≥30 ng/mL at the 6-month follow-up visit. The effect of vitamin D₃ supplementation varied by race and was more effective and significant only in black children.

Our observations affirm the previously observed association between baseline 25(OH)D concentration and the changes that occur in response to vitamin D supplementation (20, 21). There was a greater 2-month increase in 25(OH)D concentration with vitamin D supplementation and a strong negative association between change in 25(OH)D concentration and baseline 25(OH)D concentration in black children compared to white children. Both observations are likely functions of the black children’s lower baseline 25(OH)D concentrations.

Black children had consistently higher concentrations of PTH than white children. Our findings of lower 25(OH)D and higher PTH concentrations at baseline in black than in white children is similar to the observations of Warden et al (22). In their cross-sectional study of 10- to 13-year-old pubertal black children and white children, PTH was positively associated with bone strength at the tibial diaphysis in black but not in white children, primarily due to greater bone cross-sectional area with higher PTH concentrations. This finding calls into question the benefits of maximal PTH suppression in children.

We found that the negative association between 25(OH)D and PTH was linear without evidence of plateauing, thereby calling into question the appropriateness of using 25(OH)D-PTH dynamics as a surrogate indicator for defining optimal vitamin D status in children. This finding is in agreement with the report by Hill et al (5) and affirms their conclusion that 25(OH)D-PTH association in children is linear and lacks a threshold indicative of maximal suppression of PTH. However, our failure to find an inflection in the response curve could be attributed to insufficient range of 25(OH)D concentrations, mainly the number of 25(OH)D data points above 40 ng/mL. Whether PTH levels would plateau at higher concentrations of 25(OH)D, as reported in adults (23, 24), remains uncertain. Most importantly, our longitudinal design enabled us to examine whether the 25(OH)D-PTH within-child association over time was as strong as the cross-sectional association. We found the strength of within-child association was far less. This finding implies that any 25(OH)D-PTH associations found through cross-sectional studies are largely due to unmeasured confounding and are overly biased estimates of the causal effects of vitamin D on PTH.

In our study, overall increase in serum 25(OH)D concentration after vitamin D supplementation had no effect on bone turnover. In a total of 323 9- to 13-year-old black and white American children living at 34°N (Athens, Georgia) and 40°N (West Lafayette and Indianapolis, Indiana) with baseline mean 25(OH)D concentration of 28 ng/mL, dose-dependent increases in 25(OH)D with vitamin D supplementation (0, 400, 1000, 2000, and 4000 IU/d for 12 wk) had no effect on calcium absorption (7). In the same cohort, bone turnover markers were not associated with 25(OH)D at

| Table 3. OC and CTx Concentrations by Treatment Group and Racea |

<table>
<thead>
<tr>
<th>Total Sample (n = 157)</th>
<th>Black (n = 84)</th>
<th>White (n = 73)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D Group</td>
<td>Placebo Group</td>
<td>P Value</td>
</tr>
<tr>
<td>Mean OC concentrations, ng/mL</td>
<td>Mean CTX concentrations, ng/mL</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.7 ± 0.9(78)</td>
<td>1.8 ± 0.9(79)</td>
</tr>
<tr>
<td>2-mo f/u</td>
<td>1.6 ± 0.8(72)</td>
<td>1.7 ± 1.2(64)</td>
</tr>
<tr>
<td>6-mo f/u</td>
<td>1.4 ± 0.9(69)</td>
<td>1.6 ± 0.9(63)</td>
</tr>
<tr>
<td>2 mo – baseline Δ</td>
<td>−0.13 ± 0.7</td>
<td>−0.01 ± 1.0</td>
</tr>
<tr>
<td>6 mo – baseline Δ</td>
<td>−0.29 ± 1.0</td>
<td>−0.16 ± 1.1</td>
</tr>
</tbody>
</table>

Abbreviation: f/u, follow-up. Data are shown as mean ± SD (number).

*a P values of the OC and CTX group differences are unadjusted.

b P < .05 for the difference between black children and white children in the corresponding treatment group.

c P < .01 for the difference between black children and white children in the corresponding treatment group.

d P < .0001 for the difference between black children and white children in the corresponding treatment group.
baseline or with change in 25(OH)D over 12 weeks (25). Therefore, raising 25(OH)D concentrations over 12 weeks is unlikely to increase calcium absorption or have an impact on bone turnover in children. Well-designed clinical trials with longer duration of follow-up are warranted to examine the skeletal health benefits of enhancing the vitamin D status of otherwise healthy vitamin D-deficient children.

Our observed race-related differences in bone turnover and effect of vitamin D supplementation on bone turnover are in discordance with the findings of Hill et al (25). Differences in age and pubertal status of the cohorts may explain the discordance. In addition, there is a diurnal or circadian rhythm for bone turnover markers, and because markers were obtained throughout the day, we may have missed an association (26, 27). Race-related differences in the effect of vitamin D supplementation on bone turnover need further exploration.

Strengths of our study included enrollment of substantial numbers of black children, enrollment limited to periods of reduced solar ultraviolet-B radiation, and detailed characterization of determinants of vitamin D status. Limitations included reliance on a standard dose of vitamin D₃, rather than multiple doses; lack of functional outcome measures such as calcium absorption and bone mineral density; and nonfasting PTH and bone turnover markers. Also, data regarding vitamin D-binding-protein (DBP) concentrations, DBP polymorphisms, and 1,25-dihydroxyvitamin D concentrations might have provided additional insight into the racial differences that we observed in PTH concentrations and into 25(OH)D-PTH dynamics. In a recent study, black adults had lower 25(OH)D and DBP concentrations than white adults but similar concentrations of estimated bioavailable 25(OH)D, both generally and within each quintile of PTH concentration (28).

In conclusion, vitamin D₃ supplementation with 1000 IU/d in children with mean 25(OH)D concentration <20 ng/mL failed to raise their 25(OH)D concentration to 30 ng/mL. Of the supplemented children with baseline 25(OH)D concentrations <20 ng/mL, 39% continued to

<table>
<thead>
<tr>
<th>Baseline associations</th>
<th>No. of Patients</th>
<th>r</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sample</td>
<td>154</td>
<td>0.12</td>
<td>.13</td>
</tr>
<tr>
<td>25(OH)D and log OC</td>
<td>154</td>
<td>0.13</td>
<td>.69</td>
</tr>
<tr>
<td>25(OH)D and log CTx</td>
<td>81</td>
<td>0.23</td>
<td>.038</td>
</tr>
<tr>
<td>Black</td>
<td>84</td>
<td>0.21</td>
<td>.046</td>
</tr>
<tr>
<td>25(OH)D and log OC</td>
<td>73</td>
<td>-0.27</td>
<td>.02</td>
</tr>
<tr>
<td>25(OH)D and log CTx</td>
<td>73</td>
<td>0.17</td>
<td>.15</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Change over 2 mo</th>
<th>No. of Patients</th>
<th>r</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sample</td>
<td>134</td>
<td>0.25</td>
<td>.004</td>
</tr>
<tr>
<td>Δ25(OH)D and Δlog OC</td>
<td>136</td>
<td>-0.08</td>
<td>.37</td>
</tr>
<tr>
<td>Black</td>
<td>66</td>
<td>0.18</td>
<td>.14</td>
</tr>
<tr>
<td>Δ25(OH)D and Δlog CTx</td>
<td>68</td>
<td>-0.01</td>
<td>.92</td>
</tr>
<tr>
<td>White</td>
<td>68</td>
<td>0.33</td>
<td>.006</td>
</tr>
<tr>
<td>Δ25(OH)D and Δlog CTx</td>
<td>68</td>
<td>-0.12</td>
<td>.34</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Change over 6 mo</th>
<th>No. of Patients</th>
<th>r</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sample</td>
<td>130</td>
<td>0.17</td>
<td>.052</td>
</tr>
<tr>
<td>Δ25(OH)D and Δlog OC</td>
<td>132</td>
<td>-0.014</td>
<td>.87</td>
</tr>
<tr>
<td>Black</td>
<td>63</td>
<td>0.19</td>
<td>.14</td>
</tr>
<tr>
<td>Δ25(OH)D and Δlog CTx</td>
<td>64</td>
<td>0.01</td>
<td>.93</td>
</tr>
<tr>
<td>White</td>
<td>67</td>
<td>0.13</td>
<td>.28</td>
</tr>
<tr>
<td>Δ25(OH)D and Δlog CTx</td>
<td>68</td>
<td>0.014</td>
<td>.91</td>
</tr>
</tbody>
</table>

P values <.05 are shown in bold.

a Total sample adjusted for intervention, Tanner stage, sex, and race; black or white children adjusted for intervention, Tanner stage, and sex.
b Total sample adjusted for intervention, Tanner stage, sex, race, and baseline 25(OH)D; black or white children adjusted for intervention, Tanner stage, sex, and baseline 25(OH)D.
have concentrations <20 ng/mL at the 6-month follow-up visit. These findings suggest that currently recommended daily dietary allowance of vitamin D of 600 IU (2, 29) may be inadequate for preventing vitamin D deficiency in children. Overall, vitamin D supplementation had no effect on mean concentrations of PTH, OC, and CTx. Our findings of lack of effect of vitamin D supplementation on bone turnover markers may not reflect effects on skeletal health because single measurements of bone turnover markers do not correlate with bone density in children and young adults (30). We were unable to define a threshold concentration of 25(OH)D as an indicator of vitamin D sufficiency based on the relationship between 25(OH)D and PTH because we found no plateauing effect on PTH concentration with increasing concentrations of 25(OH)D. Further study is needed to examine the clinical importance of striving for higher 25(OH)D levels in children and to ascertain whether PTH levels would plateau at higher concentrations of 25(OH)D.

Acknowledgments

We thank the participants and their parents for their commitment to this project; the University of Pittsburgh’s National Institutes of Health-funded Pediatric PittNet (practice-based research network) for enhancing our recruitment efforts in their affiliated pediatric offices in the greater Pittsburgh area and for facilitating study-related activities at the Children’s Community Pediatrics-Armstrong practice in Kittanning, PA; and the MUH Clinical and Translational Research Center nursing staff for their support. We also thank Jack L. Paradise, MD, for valuable editorial suggestions.

Address all correspondence and requests for reprints to: Kumaravel Rajakumar, MD, MS, Associate Professor of Pediatrics, University of Pittsburgh School of Medicine, Children’s Hospital of Pittsburgh of UPMC, Division of General Academic Pediatrics, 3414 Fifth Avenue, Children’s Hospital Office Building, Third Floor, Pittsburgh, PA 15213. E-mail: Kumaravel.Rajakumar@chp.edu.

This work was supported by the following grants: National Institutes of Health Grants K23HD052550 (to K.R.), K24DK062895 (to S.L.G.), P30 AG024827 (to S.L.G.), R01HL112985 (to C.G.M.), and UL1 RR024153 (to the University of Pittsburgh Clinical and Translational Research Center and Pediatric PittNet); and a Children’s Hospital of Pittsburgh Research Advisory Committee seed grant (to K.R.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author Contributions: K.R. had full access to all of the data in this study and takes responsibility for the integrity and accuracy of this work as a whole from inception to publication. Other contributions of authors included: study concept and design, K.R., S.L.G., M.F.H., J.D.-J., A.N.; acquisition of data, K.R., F.O., M.A.H.; analysis and interpretation of data, K.R., C.G.M., J.Y., D.C., S.S., M.F.H., J.B., S.L.G.; drafting of the manuscript, K.R., C.G.M., J.Y., D.C.; critical revision of the manuscript, all authors; statistical analysis, C.G.M., J.Y., S.S.; securing of funding support, K.R.; and study supervision, K.R., M.A.H., S.L.G.

Disclosure Summary: The authors have no relevant financial relationships or conflicts of interest to disclose pertaining to this manuscript.

References


A randomized trial of nutrient supplements to minimise psychiatric illness after a natural disaster.
Psych Res. 2015.
A randomised trial of nutrient supplements to minimise psychological stress after a natural disaster

Bonnie J. Kaplan a,∗, Julia J. Rucklidge b, Amy R. Romijn b, Michael Dolph a

a Department of Paediatrics, Alberta Children’s Hospital Research Institute, University of Calgary, Calgary, Canada
b Department of Psychology, University of Canterbury, Christchurch, New Zealand

Abstract

After devastating flooding in southern Alberta in June 2013, we attempted to replicate a New Zealand randomised trial that showed that micronutrient (minerals, vitamins) consumption after the earthquakes of 2010–11 resulted in improved mental health. Residents of southern Alberta were invited to participate in a study on the potential benefit of nutrient supplements following a natural disaster. Fifty-six adults aged 23–66 were randomised to receive a single nutrient (vitamin D, n = 17), a few-nutrients formula (B-Complex, n = 21), or a broad-spectrum mineral/vitamin formula (BSMV, n = 18). Self-reported changes in depression, anxiety and stress were monitored for six weeks. Although all groups showed substantial decreases on all measures, those consuming the B-Complex and the BSMV formulas showed significantly greater improvement in stress and anxiety compared with those consuming the single nutrient, with large effect sizes (Cohen’s d range 0.76–1.08). There were no group differences between those consuming the B-Complex and BSMV. The use of nutrient formulas with multiple minerals and/or vitamins to minimise stress associated with natural disasters is now supported by three studies. Further research should be carried out to evaluate the potential population benefit that might accrue if such formulas were distributed as a post-disaster public health measure.

© 2015 Published by Elsevier Ireland Ltd.

1. Introduction

When natural disasters strike, one of the first daily functions to be impaired is the ability to obtain and prepare nutritious food. Hence, at a time when stress and anxiety are elevated, the nutrition needed to maximise mental health may be in short supply.

It is logical to consider that enhancing people’s intake of minerals and vitamins (micronutrients) might be helpful for coping with natural disasters, as it has been known for centuries that a good diet can help optimise health. For example, micronutrients act as cofactors in neurotransmitter synthesis and metabolism, where they can be rate-limiting factors (Ames et al., 2002). There are at least seven randomised controlled trials (RCTs) of B-Complex and combined mineral/vitamin formulations in which improvements in depression, anxiety and stress have been demonstrated (Carroll et al., 2000; Harris et al., 2011; Kennedy et al., 2010; Lewis et al., 2013; Long and Benton, 2013a; Rucklidge et al., 2012; Schlebusch et al., 2000) although not all trials using broad-spectrum micronutrients have shown benefit for changing mood and anxiety (Cockle et al., 2000; Haskell et al., 2008, 2010). However, all the negative trials were conducted on people who had no presenting psychological/psychiatric challenge. Further, a recent meta-analysis showed a small but meaningful effect of micronutrients on stress and anxiety but not mood (Long and Benton, 2013b).

The information most directly relevant to the context of natural disasters is the series of studies conducted in Christchurch, New Zealand after the earthquakes of 2010–11. When the 7.1 magnitude earthquake hit on September 4, 2010, the Mental Health and Nutrition Research Group at the University of Canterbury was in the midst of conducting a clinical trial of a broad-spectrum mineral/vitamin formula in adults with ADHD, but some individuals had completed the trial or not started the trial and therefore were not taking it on the day of the earthquake and the following weeks. Two weeks after the earthquake, those who were taking the formula at the time of the earthquake were significantly less anxious and stressed than those not taking it (Rucklidge and Blampied, 2011; Rucklidge et al., 2011). Subsequently, when the February 22, 2011 earthquake of 6.3 magnitude struck, this research group immediately implemented a randomised trial in the general population, comparing two doses of the same formula to a B Complex formula (Rucklidge et al., 2012) previously shown to be efficacious for the treatment of stress and anxiety (Carroll et al., 2000;
Kennedy et al., 2010; Schlebusch et al., 2000). A nonrandomised group of adults from the community who did not take any micronutrients served as the control group. Those taking the nutrients showed significantly lower levels of Post-traumatic Stress Disorder (PTSD) symptoms after one month as compared with the controls, and those taking the higher dose of the broad-spectrum micronutrient formula reported greater improvement in mood and anxiety than those taking the B-Complex.

In June 2013 a combination of Rocky Mountain snowmelt plus heavy rain caused a devastating flood in southern Alberta, Canada. River flow rates more than tripled in a few hours and over 100,000 people were evacuated from their homes. As in New Zealand, a group at the local university had been studying the potential benefit of multinutrient treatment of stress and anxiety, so the flood provided an opportunity to try to replicate the New Zealand earthquake studies to determine the generalizability of those findings to natural disasters. The research design allowed us in addition to investigate whether a single nutrient (vitamin D) or broader spectrum of nutrients were comparable at reducing psychological symptoms. Three micronutrient formulas were evaluated for their impact on depression, anxiety and stress associated with the Alberta floods.

### 2. Methods

At study entry (baseline) questionnaires assessed (a) exposure to the flood, (b) overall impact of the flood on physical and emotional health, (c) diet quality, and (d) depression, anxiety and stress. Those who qualified for the study were randomly allocated to one of three treatment groups (single nutrient, B-Complex, and broad-spectrum mineral/vitamin (BSMV)). Vitamin D was selected as the single nutrient comparator because of some prior evidence based on a meta-analysis of its benefit (albeit small) for people with depressive symptoms (Rijpkema et al., 2012; Shaffer et al., 2014) as well as the generally heightened public awareness about vitamin D’s contributions to health. The factorial design allocated people in equal numbers across groups. Group assignment was based on computer-generated block randomisation (block = 5), and concealed in envelopes opened at time of randomisation. Treatment effects over six weeks were evaluated from questionnaire responses; compliance and side effects were also monitored. This study was approved by the Conjoint Health Research Ethics Board of the University of Calgary (REB13-0550) and the University of Canterbury Human Ethics Committee, and was prospectively registered with the Australia New Zealand Clinical Trial Registry (ANZCTR 12613001051730).

#### 2.1. Participants

From late 2013 to mid-2014, adults in southern Alberta were invited via social media to participate in a study on the potential benefit of nutrient supplements following a natural disaster. Interested individuals were directed to a website to view. An assistant prepared the concealed randomisation assignment prior to intake interviews; interviewers who enrolled participants opened the next sequential envelope to determine group assignment. Those not meeting criteria were directed to local resources for mental health care.

Participants had to be >18 years, whose homes were damaged by the flood. They had to have at least one score above the cut-offs of the Depression, Anxiety and Stress Scale (DASS) (Lovibond and Lovibond, 1995b), as follows: >10 (for depression), >7 (for anxiety) or >14 (for stress). They also had to be free of psychiatric medications for at least four weeks. Candidates were excluded if they reported a neurological disorder involving the central nervous system (CNS) (e.g., epilepsy), known allergies to the nutrients, pregnancy or breastfeeding, untreated or unstable thyroid disease, known abnormality of mineral metabolism (e.g., Wilson’s disease), substance dependence within the previous month, currently taking any other multivitamin/mineral, or currently taking any other medication with epilepsy), known allergies to the nutrients, pregnancy or breastfeeding, untreated psychiatric medications for at least four weeks. Candidates were excluded if they reported a neurological disorder involving the central nervous system (CNS) (e.g., epilepsy), known allergies to the nutrients, pregnancy or breastfeeding, untreated or unstable thyroid disease, known abnormality of mineral metabolism (e.g., Wilson’s disease), substance dependence within the previous month, currently taking any other multivitamin/mineral, or currently taking any other medication with epilepsy).

#### 2.2. Intervention

Those who met the inclusion criteria were randomised to one of three groups. Randomisation occurred at the intake interview, after eligibility was confirmed and the consent form was signed. All intervention formulas are Health Canada-approved and have Natural Product Numbers (NPNs). Ingredients are in Table 1.

#### 2.2.1. Vitamin D, consumed in one pill/day

This vitamin is of key importance for oxidative stress at the cellular level, and for immunity, inflammation, and muscle function (Larson-Meyer, 2013). With respect to mental health, the role of vitamin D is gaining increasing support. For instance, Maddock et al. recently reported an association between low vitamin D status and vulnerability to depression (Maddock et al., 2013). Although the use of vitamin D alone to effectively manage serious mental disorders has not been supported scientifically, there are some impressive examples of treatment benefits in individual case studies (Humble, 2010). For the current study, vitamin D (1000 IU) produced by Douglas Laboratories was used (NPN 80009658). This vitamin D is in medium-sized white pressed tablets.

#### 2.2.2. B-Complex, consumed as one capsule/day

As mentioned, improvements in depression, anxiety and stress in response to supplementation with B vitamins have been demonstrated in several RCTs. A formula produced by Douglas Laboratories was used, B-Complex with Metafolin™ (NPN 80021762). This B-Complex is in large transparent gelatin capsules.

#### 2.2.3. Broad-Spectrum Mineral/Vitamin formula (BSMV), consumed as four capsules/day

The Truehope EMP™ by Truehope Nutritional Support, Ltd. (NPN 80000383). This formula is in large transparent gelatin capsules.

#### 2.3. Outcome measures

Outcome measures were completed online by participants, at baseline and then every two weeks for the duration of the trial.

#### 2.3.1. Primary outcomes (determined a priori)

- The Depression Anxiety and Stress Scale (DASS [Lovibond and Lovibond, 1995b]) was administered at the Intake Interview and used as a baseline score.

---

### Table 1 Ingredient list of daily dose of vitamin D, B-Complex, and Broad Spectrum Mineral/Vitamin formula.

<table>
<thead>
<tr>
<th>Vitamin D, consumed in 1 pill/day</th>
<th>B-Complex, consumed in 1 capsule/day</th>
<th>Broad Spectrum, consumed in 4 capsules/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A (mcg)</td>
<td>384.0</td>
<td></td>
</tr>
<tr>
<td>Vitamin B6 (mg)</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>Vitamin B12 (mcg)</td>
<td>200.0</td>
<td></td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>133.2</td>
<td></td>
</tr>
<tr>
<td>Vitamin D (IU)</td>
<td>320.0</td>
<td></td>
</tr>
<tr>
<td>Vitamin E (mg)</td>
<td>53.6</td>
<td></td>
</tr>
<tr>
<td>Thiamine (mg)</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>Riboflavin (mg)</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Folate (mcg)</td>
<td>320.0</td>
<td></td>
</tr>
<tr>
<td>5-pantothenic acid (mg)</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>Intrinsic factor (mg)</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td>Biotin (mcg)</td>
<td>240.0</td>
<td></td>
</tr>
<tr>
<td>Niacin (mg)</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td>Chromium (mcg)</td>
<td>138.8</td>
<td></td>
</tr>
<tr>
<td>Copper (mg)</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Iodine (mcg)</td>
<td>45.2</td>
<td></td>
</tr>
<tr>
<td>Iron (mg)</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>293.2</td>
<td></td>
</tr>
<tr>
<td>Magnesium (mg)</td>
<td>133.2</td>
<td></td>
</tr>
<tr>
<td>Manganese (mg)</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Molybdenum (mcg)</td>
<td>32.0</td>
<td></td>
</tr>
<tr>
<td>Phosphorous (mg)</td>
<td>186.8</td>
<td></td>
</tr>
<tr>
<td>Potassium (mg)</td>
<td>51.2</td>
<td></td>
</tr>
<tr>
<td>Selenium (mcg)</td>
<td>45.2</td>
<td></td>
</tr>
<tr>
<td>Zinc (mg)</td>
<td>10.8</td>
<td></td>
</tr>
</tbody>
</table>

*And a proprietary blend of phenylalanine, l-methionine, citrus bioflavonoids, germanium sesquioxide, nickel, vanadium, grape seed, l-glutamine, inositol, choline bitartrate and ginkgo biloba.*
Its 42 items assess current symptom severity and scores can range from zero to 126. The participants were asked to rate each item on the scale as it applied to them over the previous week, ranging from 0 to 4 (0 being not at all). Example items include: “I felt I had nothing to look forward to” (depression), “I felt I was close to panic” (anxiety), and “I found it difficult to tolerate interruptions to what I was doing” (stress). Higher scores reflect greater impairment. Scores below 10 (for depression), 7 (for anxiety) and 14 (for stress) are considered to be within the normal range.

2.3.3. Other measures

- Traumatic Exposure Severity Scale (TESS (Elal and Slade, 2005)) was adapted for the flood experience. Its 24 items assess Resource Loss (e.g., Did you need food and water aid after the flood?). Damage to Home and Goods (e.g., Did you have to relocate because your house became structurally unsafe to live in?). Personal Harm (e.g., Were you physically injured in the flood?). Concern for Significant Others (e.g., Were any members of your family or your loved ones physically injured in the flood?) and Exposure to the Grotesque (e.g., Did you see dead bodies or body parts in the period following the flood?). The scale assesses both occurrences (range 0–24) and distress if any of the occurrences were endorsed (how distressing was this for you from 1 (not at all) to 5 (extremely); range 0–120).

- Diet quality questionnaire. The diet quality items were adapted from descriptions of a healthy eater developed by Baker et al. (2003). They defined a healthy eater as someone who eats in a balanced way, eats three meals a day, does not eat too much junk food, eats moderate amounts, and stops eating when full. These descriptions were adapted and validated by Kuijer and Boyce (2012). Participants were asked to indicate from 1 (very much worse) to 7 (very healthy) how healthy they thought their diet was. Total scores ranged from 9 to 47, with a higher score indicative of a healthier diet. In the validation study, Kuijer and Boyce (2012) showed that the questions were correlated highly with a 2-week diary report of those behaviors (correlations varied from 0.49 to 0.93). Moreover, the retrospective recall was found to be a fairly accurate estimate of the eating behaviors as reported during the diary period. The summing of the items has been used successfully in other studies such that a higher score on the summed scale indicates healthier eating behaviors (Cronbach’s alpha 0.67 (Kuijer and Boyce, 2014)). This questionnaire was completed at baseline and 6 weeks.

- Alcohol, caffeine, cigarettes and illicit drugs. Every two weeks during the trial, participants were asked to record the amount of alcohol, caffeinated beverages (coffee, tea, coke, etc.), cigarettes, and illicit drugs consumed over the previous two-week period. Responses were standardized according to approximate amount of caffeine in different types of drinks (e.g., green tea was coded as 0.5 cups, but energy drinks were coded as 2 cups) and alcohol consumption was converted to an estimate of number of standard drinks consumed.

- Sleep quality. Participants were asked to complete five items of the Pittsburgh Insomnia Rating Scale assessing sleep quality (Moula et al., 2002). Questions ask about how much they were bothered by getting to sleep, waking in the night, waking too early, not getting enough sleep and not having refreshing sleep. They were asked to rate each question from 0 (not at all bothered) to 3 (severely bothered). The responses to the five items were summed for a total score ranging from 0 to 15.

Fig. 1. CONSORT flow diagram.
The two primary outcome measures were defined a priori (DASS and CGI-I). Paired sample t-tests were used to assess change from baseline to end-of-treatment for each group. The changes from baseline to the end-of-treatment were compared between randomised groups using ANCOVA, with baseline level as the covariate. Change measures (CGI-I ratings) assessed at the end of treatment were compared using one-way ANOVA. Categorical outcomes were compared between groups using Chi-square tests with odds ratios and 95% confidence intervals. Adverse event rates were compared between treatment groups using Fisher’s exact tests. All analyses on primary and secondary measures were undertaken on an intention-to-treat (ITT) basis except for the CGI-I ratings as there were no baseline ratings to carry forward. For those participants not completing the trial, data from their final assessment were used (which may have been baseline). All tests were two-tailed, and p-values less than 0.05 were considered statistically significant. Cohen’s d (with confidence intervals) was used as a measure of effect sizes, with 0.2 being small, 0.5 being medium, and 0.8 being large.

### 3. Results

The sample consisted of 56 participants, 17 assigned to vitamin D, 21 to B-Complex and 18 to BSMV (see Fig. 1 for the CONSORT diagram and Table 2 for demographic information). Baseline comparisons between groups showed no significant differences for any demographic characteristics.

There were no differences in treatment-emergent adverse events across groups (data not shown). Five people from the vitamin D group, 2 from the B-Complex group, and 4 from the BSMV group dropped out at some point prior to the sixth week of the trial. The reasons for dropout varied widely, from a cancer diagnosis to being too busy with flood recovery projects, with no apparent trends between treatment groups. Overall, compliance in taking the nutrients was high for all three treatment groups, with the vitamin D, B-Complex, and BSMV groups reporting 93%, 94%, and 93% compliance, respectively.

#### 3.1. Baseline comparisons

There were no significant group differences in DASS, IES-R or TESS scores at baseline. The DASS was also assessed at the screening stage: screening and baseline scores did not differ, indicating that the passage of time during the two weeks prior to the trial had little effect (data not shown).

#### 3.2. Primary outcomes

All groups changed significantly from pre- to post-treatment except on DASS Anxiety where the vitamin D group did not show a significant change across time. ANCOVA tests controlling for baseline scores compared the DASS change over time between groups (Table 3), where significant differences for DASS Anxiety ($F(2, 52)=5.41, p<0.01$), DASS Stress ($F(2, 52)=3.81, p<0.05$), and DASS Total ($F(2, 52)=4.33, p<0.05$) were observed. Post-hoc analysis of the change scores revealed that the B-Complex and BSMV groups improved significantly more than the vitamin D group, with no significant differences observed between B-Complex and BSMV. Overall, large effect sizes were observed on both the Anxiety and Stress subscales of the DASS and DASS Total between the vitamin D group and the B-Complex group (Anxiety, $d=0.88$; Stress, $d=0.76$; DASS Total, $d=0.81$), and between the vitamin D group and the BSMV group (Anxiety, $d=1.08$; Stress, $d=0.88$; DASS Total, $d=0.94$), indicating that the treatment effect on both the B-Complex and BSMV groups was greater than the effect of the treatment on the vitamin D group. The negligible effect sizes between the B-Complex group and the BSMV group show that the treatments were equally effective for these two groups. Fig. 2 illustrates the differential effect of the treatments across the three groups on one of the primary outcome variables, the DASS stress subscale.

CGI-I results for change in mood, anxiety, stress, sleep, and energy are shown in Table 4. Group differences were observed only for CGI stress ($F(2, 42)=3.680, p<0.05$), with post-hoc analyses revealing that the B-Complex and BSMV groups did not differ from each other but both reported greater improvements in stress ($p<0.05$) compared with the vitamin D group, with large effect sizes ($d=0.79$ and 1.09 respectively). Responders are typically classified dichotomously as those who identify themselves as “much” to “very much” improved: 2 (17%) of those in the vitamin D group, 8 (42%) of those in the B-Complex group and 8 (57%) of those in the BSMV group were classified as responders on the CGI stress scale, with post-hoc analyses revealing that significantly more people were classified as responders in the BSMV group compared with the vitamin D group ($\chi^2 (n=26)=4.473, p<0.05$; odds ratio=6.7 (95%CI=1.047–42.431)).

#### 3.3. Secondary outcomes

There were significant changes for all the groups on the IES-R...
3.4. Other variables

Additional analyses were performed on changes in diet quality and behavior throughout the study. There were no significant changes reported for cannabis, alcohol, tobacco (smoking) or caffeine consumption from baseline to week 6 for any of the groups and no differences between groups. There were no group differences at end of treatment in reported changes in exercise over the trial. Self-reported diet quality improved over the 6 weeks in all groups, but there were no differences between groups either at baseline or end of treatment. Additionally, improved sleep quality was observed from baseline to week 6 only for the BSMV group (r (1, 12) = 4.214, p < 0.001), but there were no group differences in sleep quality at the end of the trial.

4. Discussion

This trial found that people consuming the B-Complex or the BSMV formulas showed significantly greater reduction in stress and anxiety compared with those consuming only vitamin D. Based on the primary and secondary outcome measures, the groups consuming the B-Complex or the BSMV formulas showed significant changes on all variables from beginning to end of treatment. The vitamin D group showed significant changes on all variables except the Anxiety subscale of the DASS and the Arousal subscale of the IES-R. Significant group differences were observed showing greater change in self-reported Anxiety and Stress for vitamin D vs. BSMV (effect size: 0.37–0.81) and anxiety vs. BSMV (effect size: 0.37–0.70) (Table 3).

### Table 3

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Treatment</th>
<th>Baseline</th>
<th>Change, baseline to 6 weeks</th>
<th>Percent change</th>
<th>p for within group change</th>
<th>Post-hoc analysis</th>
<th>p for time by treatment interaction</th>
<th>B-Complex, BSMV vs. Vitamin D (95% CI)</th>
<th>Vitamin D vs. BSMV (effect size)</th>
<th>Vitamin D vs. Vitamin D (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary outcome depression</td>
<td>Vitamin D</td>
<td>11.8 (7.3)</td>
<td>–2.8 (3.8)</td>
<td>–24%</td>
<td>0.008</td>
<td>0.135</td>
<td>–</td>
<td>0.58 (0.06 to 1.24)</td>
<td>0.64 (0.04 to 1.32)</td>
<td>0.05 (0.58 to 1.32)</td>
</tr>
<tr>
<td></td>
<td>B-Complex</td>
<td>13.0 (8.5)</td>
<td>–6.7 (6.8)</td>
<td>–52%</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BSMV</td>
<td>14.3 (8.2)</td>
<td>–7.6 (8.8)</td>
<td>–54%</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vitamin D</td>
<td>11.8 (7.0)</td>
<td>–2.4 (5.6)</td>
<td>–20%</td>
<td>0.104</td>
<td>0.007</td>
<td>0.007</td>
<td>0.007</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>Anxiety</td>
<td>B-Complex</td>
<td>8.4 (6.2)</td>
<td>–5.1 (4.9)</td>
<td>–61%</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BSMV</td>
<td>8.7 (7.2)</td>
<td>–6.1 (7.5)</td>
<td>–70%</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vitamin D</td>
<td>21.1 (6.6)</td>
<td>–5.6 (5.9)</td>
<td>–27%</td>
<td>0.026</td>
<td>0.026</td>
<td>0.026</td>
<td>0.026</td>
<td>0.026</td>
<td></td>
</tr>
<tr>
<td>Stress</td>
<td>B-Complex</td>
<td>18.6 (8.7)</td>
<td>–10.1 (7.5)</td>
<td>–54%</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BSMV</td>
<td>20.0 (9.9)</td>
<td>–11.7 (10.1)</td>
<td>–59%</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vitamin D</td>
<td>44.7 (17.1)</td>
<td>–10.8 (16.7)</td>
<td>–24%</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B-Complex</td>
<td>40.0 (21.2)</td>
<td>–21.8 (17.1)</td>
<td>–55%</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BSMV</td>
<td>43.0 (21.5)</td>
<td>–25.4 (23.8)</td>
<td>–59%</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Secondary outcome IES-R avoid</td>
<td>Vitamin D</td>
<td>1.5 (0.6)</td>
<td>–0.5 (0.8)</td>
<td>–33%</td>
<td>0.036</td>
<td>0.607</td>
<td>–</td>
<td>0.36 (0.27 to 0.99)</td>
<td>0.30 (0.17 to 0.96)</td>
<td>0.06 (0.56 to 0.96)</td>
</tr>
<tr>
<td></td>
<td>B-Complex</td>
<td>1.2 (0.7)</td>
<td>–0.5 (0.5)</td>
<td>–42%</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BSMV</td>
<td>1.2 (0.7)</td>
<td>–0.4 (0.6)</td>
<td>–33%</td>
<td>0.016</td>
<td>0.016</td>
<td>0.016</td>
<td>0.016</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>IES-R intrusion</td>
<td>Vitamin D</td>
<td>1.7 (0.7)</td>
<td>–0.7 (0.6)</td>
<td>–41%</td>
<td>0.001</td>
<td>0.278</td>
<td>–</td>
<td>0.45 (0.19 to 1.11)</td>
<td>0.48 (0.19 to 1.15)</td>
<td>0.03 (0.60 to 1.15)</td>
</tr>
<tr>
<td></td>
<td>B-Complex</td>
<td>1.7 (0.7)</td>
<td>–0.6 (0.6)</td>
<td>–43%</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BSMV</td>
<td>1.4 (0.7)</td>
<td>–0.6 (0.6)</td>
<td>–43%</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>IES-R arousal</td>
<td>Vitamin D</td>
<td>1.7 (0.5)</td>
<td>–0.4 (0.9)</td>
<td>–24%</td>
<td>0.072</td>
<td>0.132</td>
<td>–</td>
<td>0.39 (0.24 to 1.04)</td>
<td>0.50 (0.17 to 1.18)</td>
<td>0.11 (0.52 to 1.18)</td>
</tr>
<tr>
<td></td>
<td>B-Complex</td>
<td>1.4 (0.8)</td>
<td>–0.6 (0.8)</td>
<td>–43%</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BSMV</td>
<td>1.3 (0.6)</td>
<td>–0.6 (0.7)</td>
<td>–46%</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>IES-R total</td>
<td>Vitamin D</td>
<td>36.3 (9.8)</td>
<td>–9.7 (16.3)</td>
<td>–27%</td>
<td>0.026</td>
<td>0.323</td>
<td>–</td>
<td>0.50 (0.15 to 0.95)</td>
<td>0.40 (0.09 to 0.73)</td>
<td>0.09 (0.53 to 0.73)</td>
</tr>
<tr>
<td></td>
<td>B-Complex</td>
<td>31.6 (14.1)</td>
<td>–13.6 (10.9)</td>
<td>–43%</td>
<td>0.001</td>
<td>0.155</td>
<td>–</td>
<td>0.50 (0.17 to 1.15)</td>
<td>0.40 (0.17 to 1.10)</td>
<td>0.11 (0.52 to 1.10)</td>
</tr>
<tr>
<td></td>
<td>BSMV</td>
<td>28.2 (12.0)</td>
<td>–11.4 (11.6)</td>
<td>–40%</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>

* Using ITT protocol. Vitamin D (n = 17), B-Complex (n = 21), BSMV (n = 18) DASS—Depression Anxiety and Stress Scale; IES-R—Impact of Event Scale; BSMV—Broad Spectrum Mineral/Vitamin formula

b ANCOVA comparing changes from baseline to 6 weeks across the three treatment groups, controlling for baseline score

b B-Complex, BSMV vs. Vitamin D indicates that B-Complex and BSMV had a greater change than Vitamin D

effect size as Cohen’s d: mean difference between the estimated marginal means/mean squared error corrected for unequal group sizes

---

Fig. 2. Change in DASS Stress from baseline to week 6. BSMV = Broad Spectrum Mineral Vitamin.

except for vitamin D on the arousal subscale (Table 3). There were no group differences across time on the IES-R.

3.4. Other variables

Additional analyses were performed on changes in diet quality and behavior throughout the study. There were no significant changes reported for cannabis, alcohol, tobacco (smoking) or caffeine consumption from baseline to week 6 for any of the groups and no differences between groups. There were no group differences at end of treatment in reported changes in exercise over the trial. Self-reported diet quality improved over the 6 weeks in all groups, but there were no differences between groups either at baseline or end of treatment. Additionally, improved sleep quality was observed from baseline to week 6 only for the BSMV group (r (1, 12) = 4.214, p < 0.001), but there were no group differences in sleep quality at the end of the trial.

Please cite this article as: Kaplan, B.J., et al., A randomised trial of nutrient supplements to minimise psychological stress after a natural disaster. Psychiatry Research (2015), http://dx.doi.org/10.1016/j.psychres.2015.05.080
B.J. Kaplan et al. / Psychiatry Research (2015), http://dx.doi.org/10.1016/j.psychres.2015.05.080

Table 4
Change in mood, sleep, anxiety, stress, and energy at week 6, by treatment group from the CGI-I scale.a

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Vitamin D (n=12) M (SD)</th>
<th>B-Complex (n=19) M (SD)</th>
<th>BSMV (n=14) M (SD)</th>
<th>p for treatment comparisonb</th>
<th>Post-hoc analysesb</th>
<th>Vit D vs. B-complex (effect sizec (95% CI))</th>
<th>Vit D vs. BSMV (effect sized (95% CI))</th>
<th>B-complex vs. BSMV (effect sized (95% CI))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGI-I change in:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mood</td>
<td>3.0 (1.0)</td>
<td>2.5 (1.0)</td>
<td>3.0 (1.4)</td>
<td>0.370</td>
<td>–</td>
<td>0.37 (–0.37 to 1.10)</td>
<td>0 (–0.78 to 0.78)</td>
<td>0.36 (–0.35 to 1.08)</td>
</tr>
<tr>
<td>Anxiety</td>
<td>3.1 (0.9)</td>
<td>2.7 (1.0)</td>
<td>2.7 (0.8)</td>
<td>0.467</td>
<td>–</td>
<td>0.42 (–0.32 to 1.16)</td>
<td>0.46 (–0.33 to 1.26)</td>
<td>0.04 (–0.08 to 0.74)</td>
</tr>
<tr>
<td>Stress</td>
<td>3.4 (1.1)</td>
<td>2.7 (0.9)</td>
<td>2.5 (0.7)</td>
<td>0.034</td>
<td>B-Complex, BSMV &gt; Vitamin D</td>
<td>0.78 (0.03 to 1.54)</td>
<td>1.09 (0.24 to 1.93)</td>
<td>0.29 (0.43 to 1.01)</td>
</tr>
<tr>
<td>Energy</td>
<td>3.3 (1.2)</td>
<td>3.2 (0.9)</td>
<td>2.9 (0.9)</td>
<td>0.584</td>
<td>–</td>
<td>0.03 (–0.70 to 0.76)</td>
<td>0.49 (–0.31 to 1.29)</td>
<td>0.46 (–0.26 to 1.18)</td>
</tr>
<tr>
<td>Sleep</td>
<td>3.4 (1.1)</td>
<td>3.4 (0.8)</td>
<td>3.2 (0.6)</td>
<td>0.807</td>
<td>–</td>
<td>0.03 (–0.70 to 0.76)</td>
<td>0.23 (–0.56 to 1.01)</td>
<td>0.19 (–0.52 to 0.91)</td>
</tr>
</tbody>
</table>

BSMV, Broad Spectrum Mineral/Vitamin formula.

a Data are means and standard deviations (SD) from the CGI-I only from people who completed the week 6 survey.
b Results from ANOVA model.
c A lower score is indicative of greater improvement on this scale (which ranges from 1 very much improved to 7 very much worse with 4 indicative of no change); hence, B-Complex, BSMV > Vitamin D means that the B-Complex and BSMV groups reported greater improvement compared with the Vitamin D group.
d Cohen’s d calculated as difference between two groups/pooled standard deviation.

both the BSMV and B-Complex groups as compared with the vitamin D group. Effect sizes between these groups were large, and there were no differences between the BSMV group and the B-Complex group. Group differences could not be better accounted for by changes in alcohol, cigarette and caffeine consumption, exercise, diet, or sleep.

This replication of the NZ earthquake studies (Rucklidge et al., 2011, 2012; Rucklidge and Blampied, 2011) suggests the possibility that micronutrients could be useful for the reduction and prevention of mental health problems following natural disasters and that a greater spectrum of nutrients is more effective than one nutrient alone. Although the measure of dietary quality employed in this study was a general one, and did not assess actual nutrient intake, the results are consistent with what would be expected: that nutrient intake in people displaced from their homes would decrease from the crisis but would increase over time as they returned to their normal life patterns. The demonstration that more powerful effects on psychological functioning can be achieved with a greater number of nutrients challenges the typical approach of giving only one nutrient to effect symptom change (Rucklidge et al., 2013). The findings reported here are also consistent with other studies showing that nutrient treatment with B-Complex or broad-spectrum formulas has a positive impact on mental health (Kaplan et al., 2007; Rucklidge and Kaplan, 2013).

Though this was a replication, the issue of PTSD could not be addressed, as the IES-R scores at baseline were much lower than those obtained in the earthquake study with many fewer having probable PTSD at baseline. The experience of earthquakes and floods are not the same. As with most floods, the one studied here was a single event, followed by an outpouring of civic assistance. In contrast, the NZ earthquakes lasted for over a year, with participants experiencing 1–2 significant aftershocks every day through the trial (source: www.geonet.co.nz).

This study’s main limitation was the difficulty in recruiting, which resulted in a small sample size. One cause of that difficulty was that the civic support provided by the government included mental health treatment that usually resulted in medication, an exclusion criterion for this study. On the other hand, the sample size meant that only large effects between groups could be detected. The large effect sizes observed when compared to the single nutrient formula highlights the potential value of consuming either a B-Complex or broader micronutrient formula for improving public health following an environmental catastrophe. The fact that both of these formulas provide many B vitamins adds to the evidence showing the value of B vitamins in managing stress.

The BSMV formulation has three non-nutrient, botanical components: ginkgo biloba, citrus bioflavonoids, and grape seed. They are present because the manufacturer believes they can benefit general brain health, but their quantities are so tiny relative to those used elsewhere both clinically and in research that it is unlikely they would have a significant impact on mental health on their own. For instance, one study of ginkgo biloba’s effect on anxiety used doses 10–20 times higher than the dose in the BSMV formulation (Woelk et al., 2007); we are unaware of studies on the effects of citrus bioflavonoids or grape seed for mental health, although doses suggested clinically for these botanicals are much higher than the doses contained in the BSMV.

The results need to be interpreted bearing in mind the lack of placebo (the use of which would have been unethical) and the lack of blinding. Taking four capsules may induce a more powerful placebo effect than taking one, although the B-Complex was only one capsule and did induce the same changes as taking four. Generalizability is limited by our recruitment method through social media, meaning that we may not have reached those people who did not have access to the Internet either due to lower socioeconomic status or through greater displacement after the flood. Another study limitation was that our measure of food intake could not capture the intake of specific nutrients. Analysis of nutrients from food diaries could be considered in future research if participants are able to provide additional information, but this could be a challenge in a post-disaster crisis setting. Such additional data could inform whether the dietary intake of the participants after the flood was inadequate or whether the nutritional requirements of the body following the flood increased to respond to the additional stress. The latter hypothesis would be consistent with the triage theory proposed by McCann and Ames (2009) that states that when the “availability of a micronutrient is inadequate, nature ensures that micro-nutrient-dependent functions required for short-term survival are protected at the expense of functions whose lack has only longer-term consequences” page 889. In other words, nutrients are triaged to the flight-flight response, and in so doing, optimal brain function may be compromised, leading to the expression of psychological symptoms.

Natural disasters pose a significant public health challenge. Additional nutrients provide the body and brain with what is needed to cope with chronic stress at a time when nutrient intake is compromised, given how metabolically demanding the stress response can be if sustained over time (McCann and Ames, 2009). Micronutrient supplements have consistently been shown to benefit mental health, and they would be a cheap and easy intervention to add to all crisis teams (Ames et al., 2002). Further international research on this intervention is suggested.
Author contributions

BJK directed the study, interpreted results, and wrote the first draft of the manuscript; JJR directed the analysis and interpretation of the results; AR established all data collection procedures to be parallel to the study being replicated, and interpreted the results; MD coordinated the study, supervised the interviewers, and managed the data entry and analyses. All authors edited the final version of the manuscript.

Conflict of interest

No author has any conflict of interest or financial relationship to report.

Acknowledgments

This research was funded by a University of Calgary private donor fund. We thank the two companies who donated the nutrients: Douglas Laboratories (who provided the vitamin D and B-Complex) and Truehope Nutritional Support (who supplied the B-Complex) and Truehope Nutritional Support (who supplied the Truehope EMPTM). We thank our study interviewers Marsha Carnt, Amanda Loven, Marcus Kupila, Wendy Salvisberg, and Lida van den Hadelkamp. We also express our gratitude to the participants, who joined this study during a very stressful time in their lives.

References


Please cite this article as: Kaplan, B.J., et al., Randomised trial of nutrient supplements to minimise psychological stress after a natural disaster. Psychiatry Research (2015), http://dx.doi.org/10.1016/j.psychres.2015.05.080
Oral High-Dose Multivitamins and Minerals After Myocardial Infarction
A Randomized Trial
Gervasio A. Lamas, MD; Robin Boineau, MD, MA; Christine Goertz, DC, PhD; Daniel B. Mark, MD, MPH; Yves Rosenberg, MD; Mario Stylianou, PhD; Theodore Rozema, MD; Richard L. Nahin, PhD, MPH; Lauren Lindblad, MS; Eldrin F. Lewis, MD; Jeanne Drisko, MD; and Kerry L. Lee, PhD, for the TACT (Trial to Assess Chelation Therapy) Investigators*

Background: Whether high-dose multivitamins are effective for secondary prevention of atherosclerotic disease is unknown.

Objective: To assess whether oral multivitamins reduce cardiovascular events and are safe.

Design: Double-blind, placebo-controlled, 2 × 2 factorial, multicenter, randomized trial. (ClinicalTrials.gov: NCT00044213)

Setting: 134 U.S. and Canadian academic and clinical sites.

Patients: 1708 patients aged 50 years or older who had myocardial infarction (MI) at least 6 weeks earlier and had serum creatinine levels of 176.8 μmol/L (2.0 mg/dL) or less.

Intervention: Patients were randomly assigned to an oral, 28-component, high-dose multivitamin and multimineral mixture or placebo.

Measurements: The primary end point was time to total death, recurrent MI, stroke, coronary revascularization, or hospitalization for angina.

Results: The median age was 65 years, and 18% of patients were women. The qualifying MI occurred a median of 4.6 years (interquartile range [IQR], 1.6 to 9.2 years) before enrollment. Median follow-up was 55 months (IQR, 26 to 60 months). Patients received vitamins for a median of 31 months (IQR, 13 to 59 months) in the vitamin group and 35 months (IQR, 13 to 60 months) in the placebo group (P = 0.65). Totals of 645 (76%) and 646 (76%) patients in the vitamin and placebo groups, respectively, completed at least 1 year of oral therapy (P = 0.98), and 400 (47%) and 426 (50%) patients, respectively, completed at least 3 years (P = 0.23). Totals of 394 (46%) and 390 (46%) patients in the vitamin and placebo groups, respectively, discontinued the vitamin regimen (P = 0.67), and 17% of patients withdrew from the study. The primary end point occurred in 230 (27%) patients in the vitamin group and 253 (30%) in the placebo group (hazard ratio, 0.89 [95% CI, 0.75 to 1.07]; P = 0.21). No evidence suggested harm from vitamin therapy in any category of adverse events.

Limitation: There was considerable nonadherence and withdrawal, limiting the ability to draw firm conclusions (particularly about safety).

Conclusion: High-dose oral multivitamins and multiminerals did not statistically significantly reduce cardiovascular events in patients after MI who received standard medications. However, this conclusion is tempered by the nonadherence rate.

Primary Funding Source: National Institutes of Health.


* TACT Investigators are listed in the Appendix, available at www.annals.org.

Patients who maintain a diet rich in a highly complex mix of antioxidants and other micronutrients have lower rates of atherosclerosis (1–3). Clinical trials testing isolated and combination oral micronutrients have not replicated these benefits. Recent meta-analyses noted that only vitamins A, C, and E and the antioxidant mineral selenium have been tested in well-designed trials, with mixed results: High doses of vitamins A and E might increase risk for cancer in selected patients, vitamin C was inactive, and selenium might be beneficial (4, 5). Yet, studies of a few vitamins and minerals do not fully reflect the supplement use of a large segment of the U.S. population, which increasingly favors multivitamin and multiminer al supplements.

TACT (Trial to Assess Chelation Therapy), a 2 × 2 factorial trial funded by the National Heart, Lung, and Blood Institute and the National Center for Complementary and Alternative Medicine (6, 7), assessed whether an EDTA-based chelation regimen or an oral high-dose multivitamin and multiminer al supplement improved cardiovascular outcomes and was effective for secondary prevention in patients with a history of cardiovascular disease. The chelation results have been published (8). This article compares oral multivitamins and multiminer als with placebo.

Methods
Design
This double-blind, 2 × 2 factorial trial randomly assigned patients to receive oral vitamins and intravenous chelation infusions, oral placebo and intravenous chelation infusions, oral vitamins and placebo intravenous infusions, and oral placebo and placebo intravenous infusions. The design and organizational aspects of TACT have been published (7). The institutional review board at each clinical site approved the study, and patients provided written informed consent. A data and safety monitoring board monitored the study.

See also:
Print
Related articles ......................... 806, 824
Editorial comment ...................... 850
Summary for Patients ................. I-20
Web-Only
Supplement
Multivitamins and Minerals After Myocardial Infarction

Original Research

Although high-dose vitamins and minerals are commonly used, whether they reduce cardiovascular events after myocardial infarction (MI) is not known.

Contribution
In this randomized trial, time to recurrent MI, stroke, coronary revascularization, hospitalization for angina, or death did not differ among participants who received daily high-dose multivitamins and minerals or placebo.

Caution
Many patients in the placebo and multivitamin groups withdrew from the trial or did not adhere to treatments during the study, which lasted several years.

Implication
High-dose multivitamins and minerals do not seem to be useful for secondary prevention of cardiovascular events after MI.

—The Editors

Setting and Participants
Eligible patients were at least 50 years of age and had sustained myocardial infarction (MI) 6 weeks or more before enrollment. Patients were ineligible if they were women of childbearing age, had a serum creatinine level greater than 176.8 μmol/L (>2.0 mg/dL), or had other exclusion criteria as previously reported (7). Patients were enrolled at 134 sites in the United States and Canada (Figure 1).

Randomization and Interventions
Oral vitamins and placebo were prepared by the vitamin manufacturers and shipped to the central pharmacy for distribution to the sites. The active high-dose vitamin treatment was a 28-component mixture to be administered as 3 caplets twice daily throughout the trial, designed to reflect the vitamin regimen commonly used by chelation practitioners (Table 1). The placebo caplets contained methylcellulose filler. Intravenous treatment consisted of 40 infusions of disodium EDTA-based chelation therapy or a normal saline placebo (7, 9). The vitamins and infusion therapy were double-blinded. During the infusion phase, all patients received an open-label, oral, low-dose vitamin regimen.

Outcomes and Follow-up
The primary end point was a composite of time to death from any cause, reinfarction, stroke, coronary revascularization, or hospitalization for angina. The composite of time to cardiovascular death, reinfarction, or stroke was a prespecified secondary end point. A blinded independent clinical events committee at Brigham and Women’s Hospital, Boston, Massachusetts, adjudicated all nonprocedural components of the primary end point. The Duke Clinical Research Institute, Durham, North Carolina, verified coronary revascularizations from the source medical record.

Patients were seen at baseline and each chelation infusion visit. After the infusion phase, patients were called quarterly; attended annual clinic visits; and were seen at the end of the trial or at the 5-year follow-up, whichever was first. Vitamin or placebo caplets were distributed every 3 to 6 months. Unused pills were returned to the site to assess adherence.

Safety monitoring included periodic physical examinations and laboratory assessments. A blinded medical monitor at the Duke Clinical Research Institute reviewed all

### Figure 1. Study flow diagram.

- Patients assessed for eligibility (n = 1850)
- Patients excluded (n = 147)
- Patients randomly assigned (n = 1708)
- Randomly assigned to receive high-dose vitamins (n = 853)
  - Received the vitamins for ≥1 y: 645
  - Received the vitamins for ≥3 y: 400
  - Never received the vitamin intervention: 30
- Randomly assigned to receive placebo (n = 855)
  - Received the placebo for ≥1 y: 646
  - Received the placebo for ≥3 y: 426
  - Never received the placebo intervention: 32
- Discontinued or never started intervention (n = 394)
  - Patient declined or did not adhere: 293
  - Adverse event or effect: 39
  - Closed study site: 21
  - Physician preference: 22
  - Terminal illness or comorbid conditions: 19
  - Withdrew from the study (n = 141)
    - Lost to follow-up (n = 13)
- Discontinued or never started intervention (n = 390)
  - Patient declined or did not adhere: 304
  - Adverse event or effect: 31
  - Closed study site: 26
  - Physician preference: 19
  - Terminal illness or comorbid conditions: 10
  - Withdrew from the study (n = 148)
    - Lost to follow-up (n = 9)
- Included in primary analysis (n = 853)
- Included in primary analysis (n = 855)

* Includes 1 placebo recipient who died before starting the intervention.
† 297 high-dose vitamin recipients and 285 placebo recipients had follow-up after discontinuation and 97 high-dose vitamin recipients and 105 placebo recipients had no follow-up after discontinuation.
‡ 43 met the primary end point before withdrawal (16 in the high-dose vitamin group and 27 in the placebo group). Among the patients who had not had an event before withdrawal, 9 (5 in the high-dose vitamin group and 4 in the placebo group) were found through search of death registries to have died. All of these events were included in the primary end point analysis.
serious adverse events. We prespecified several subgroups for analyses assessing underrepresented populations, subgroups of interest, and high-risk populations (7, 8).

Statistical Analysis
We originally planned to enroll 2372 patients over 3 years with a minimum follow-up of 1 year. This number provided 85% power for detecting a 25% relative reduction in the primary end point, assuming a 2.5-year event rate in the placebo group of 20% and a significance level of 0.05. Because of difficulty enrolling patients, the blinded investigators requested, and the data and safety monitoring board and sponsors granted, a reduction in sample size to 1700 coupled with an increase in follow-up to preserve the 85% unconditional power (8).

Secure, Web-based randomization used permuted blocks stratified by clinical site. Treatment groups were compared as randomized (intention-to-treat) using 2-sided significance tests. The log-rank test (10) was used for the statistical comparison of treatment groups with respect to clinical end points. Cumulative event rates were calculated according to the Kaplan–Meier method (11). Hazard ratios with associated CIs were calculated using the Cox proportional hazards model (12). The Cox model was also used to assess the consistency of treatment effects by testing for interactions between treatment and the baseline characteristics prespecified for subgroup analysis, as well as assignment to treatment or placebo infusions.

Continuous variables are expressed as medians and interquartile ranges (IQRs) unless otherwise specified. Group comparisons of simple proportions were done using the Pearson chi-square test. Final statistical analyses were done using SAS software, versions 8.2 and 9.2 (SAS Institute, Cary, North Carolina).

During the trial, the data and safety monitoring board requested 11 interim analyses of the data. These interim reviews were done primarily to assess the safety and efficacy of the EDTA chelation regimen and used a flexible α-spending function approach with monitoring boundaries similar to the O’Brien–Fleming method (13, 14). The level of significance required for the primary analysis at the completion of the study was 0.036.

Role of the Funding Source
The National Heart, Lung, and Blood Institute and the National Center for Complementary and Alternative Medicine of the National Institutes of Health provided funding or had a role in the execution, interpretation, or decision to submit the manuscript for publication.

RESULTS
Between 10 September 2003 and 4 October 2010, a total of 1708 patients were randomly assigned: 853 to the high-dose vitamin group and 855 to the placebo group.

Table 1. Vitamin Components

<table>
<thead>
<tr>
<th>High-Dose Regimen*</th>
<th>Total Amount for 6 Pills</th>
<th>Daily Value, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A (as fish liver oil and β-carotene)</td>
<td>25 000 IU</td>
<td>500</td>
</tr>
<tr>
<td>Vitamin C (as ascorbic acid, magnesium ascorbate, and potassium ascorbate)</td>
<td>1200 mg</td>
<td>2000</td>
</tr>
<tr>
<td>Vitamin D2 (as cholecalciferol)</td>
<td>100 IU</td>
<td>25</td>
</tr>
<tr>
<td>Vitamin E (as dl-α-tocopheryl succinate and dl-α-tocopheryl acetate)</td>
<td>400 IU</td>
<td>1333</td>
</tr>
<tr>
<td>Vitamin K1 (as phylloquinone)</td>
<td>60 μg</td>
<td>75</td>
</tr>
<tr>
<td>Thiamin (vitamin B1, as thiamin mononitrate)</td>
<td>100 mg</td>
<td>6667</td>
</tr>
<tr>
<td>Niacin (as niacinamide and niacin)</td>
<td>200 mg</td>
<td>1000</td>
</tr>
<tr>
<td>Vitamin B6 (as pyridoxine hydrochloride)</td>
<td>50 mg</td>
<td>2500</td>
</tr>
<tr>
<td>Folate (as folate)</td>
<td>800 μg</td>
<td>200</td>
</tr>
<tr>
<td>Vitamin B12 (as cyanocobalamin)</td>
<td>100 μg</td>
<td>1667</td>
</tr>
<tr>
<td>Biotin</td>
<td>300 μg</td>
<td>100</td>
</tr>
<tr>
<td>Pantothenic acid (as d-calcium pantothenate)</td>
<td>400 mg</td>
<td>4000</td>
</tr>
<tr>
<td>Calcium (as calcium citrate and calcium ascorbate)</td>
<td>500 mg</td>
<td>50</td>
</tr>
<tr>
<td>Iodine (from kelp)</td>
<td>150 μg</td>
<td>100</td>
</tr>
<tr>
<td>Magnesium (as magnesium aspartate, magnesium ascorbate, and magnesium amino acid chelate)</td>
<td>500 mg</td>
<td>125</td>
</tr>
<tr>
<td>Zinc (as zinc amino acid chelate)</td>
<td>20 mg</td>
<td>133</td>
</tr>
<tr>
<td>Selenium (as selenium amino acid chelate)</td>
<td>200 μg</td>
<td>286</td>
</tr>
<tr>
<td>Copper (as copper amino acid chelate)</td>
<td>2 mg</td>
<td>100</td>
</tr>
<tr>
<td>Manganese (as manganese amino acid chelate)</td>
<td>20 mg</td>
<td>400</td>
</tr>
<tr>
<td>Chromium (as chromium polynicotinate)</td>
<td>200 μg</td>
<td>167</td>
</tr>
<tr>
<td>Molybdenum (as molybdenum amino acid chelate)</td>
<td>150 μg</td>
<td>200</td>
</tr>
<tr>
<td>Potassium (as potassium aspartate and potassium ascorbate)</td>
<td>99 mg</td>
<td>3</td>
</tr>
<tr>
<td>Choline (as choline bitartrate)</td>
<td>150 mg</td>
<td>–†</td>
</tr>
<tr>
<td>Inositol</td>
<td>50 mg</td>
<td>–†</td>
</tr>
<tr>
<td>PABA (as paraaminobenzoic acid)</td>
<td>50 mg</td>
<td>–†</td>
</tr>
<tr>
<td>Boron (as boron aspartate and boron citrate)</td>
<td>2 mg</td>
<td>–†</td>
</tr>
<tr>
<td>Vanadium (as vanadyl sulfate)</td>
<td>39 μg</td>
<td>–†</td>
</tr>
<tr>
<td>Zinc/citrate complex</td>
<td>100 mg</td>
<td>–†</td>
</tr>
</tbody>
</table>

* Three pills twice daily. Other ingredients were croscarmellose sodium, microcrystalline cellulose, magnesium stearate, hydroxypropyl cellulose, and silicon dioxide.
† Daily value not established.

The last follow-up visit was on 31 October 2011. The median follow-up was 55 months (IQR, 26 to 60 months). The trial ended after the protocol-specified enrollment and follow-up and was not stopped on the basis of interim analyses.

Baseline Characteristics
Baseline characteristics were similar between the groups (Table 2). Median age was 65 years (IQR, 59 to 72 years), 18% were women, and 6% were nonwhite. The qualifying MI had occurred a median of 4.6 years (IQR, 1.6 to 9.2 years) before enrollment. The study population had a high incidence of diabetes (31%), previous coronary revascularizations (83%), and use of medications recommended by guidelines. Patients had a median fasting low-density lipoprotein cholesterol level of 2.30 mmol/L (89 mg/dL) (IQR, 1.73 to 2.98 mmol/L [67 to 115 mg/dL]).
Table 2. Baseline Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>High-Dose Vitamin Group (n = 853)</th>
<th>Placebo Group (n = 855)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median age (IQR), y</td>
<td>65 (59–72)</td>
<td>65 (60–72)</td>
</tr>
<tr>
<td>Women, n (%)</td>
<td>147 (17)</td>
<td>152 (18)</td>
</tr>
<tr>
<td>White, n (%)</td>
<td>797 (93)</td>
<td>808 (95)</td>
</tr>
<tr>
<td>Hispanic, n (%)</td>
<td>20 (2)</td>
<td>31 (4)</td>
</tr>
<tr>
<td>Median BMI (IQR), kg/m²</td>
<td>29 (26–33)</td>
<td>30 (27–34)</td>
</tr>
<tr>
<td>Median SBP (IQR), mm Hg</td>
<td>130 (118–140)</td>
<td>130 (120–140)</td>
</tr>
<tr>
<td>Median DBP (IQR), mm Hg</td>
<td>76 (70–81)</td>
<td>76 (70–80)</td>
</tr>
<tr>
<td><strong>History</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypercholesterolemia, n (%)</td>
<td>680 (81)</td>
<td>690 (82)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>574 (67)</td>
<td>595 (70)</td>
</tr>
<tr>
<td>Former cigarette smoker, n (%)</td>
<td>487 (57)</td>
<td>486 (55)</td>
</tr>
<tr>
<td>Angina pectoris, n (%)</td>
<td>447 (52)</td>
<td>479 (56)</td>
</tr>
<tr>
<td>Anterior MI, n (%)</td>
<td>341 (40)</td>
<td>333 (39)</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>282 (33)</td>
<td>256 (30)</td>
</tr>
<tr>
<td>Congestive heart failure, n (%)</td>
<td>137 (16)</td>
<td>170 (20)</td>
</tr>
<tr>
<td>Peripheral vascular disease, n (%)</td>
<td>125 (15)</td>
<td>143 (17)</td>
</tr>
<tr>
<td>Valvular heart disease, n (%)</td>
<td>72 (9)</td>
<td>103 (12)</td>
</tr>
<tr>
<td>Atrial fibrillation, n (%)</td>
<td>80 (10)</td>
<td>115 (14)</td>
</tr>
<tr>
<td>Stroke, n (%)</td>
<td>56 (7)</td>
<td>55 (6)</td>
</tr>
<tr>
<td>Median time from qualifying MI to randomization (IQR), y</td>
<td>4.5 (1.6–9.5)</td>
<td>4.6 (1.7–9.0)</td>
</tr>
<tr>
<td>CABG or PCI, n (%)</td>
<td>705 (83)</td>
<td>709 (83)</td>
</tr>
<tr>
<td>PCI, n (%)</td>
<td>484 (57)</td>
<td>523 (61)</td>
</tr>
<tr>
<td>CABG, n (%)</td>
<td>390 (46)</td>
<td>384 (45)</td>
</tr>
<tr>
<td><strong>Concomitant medications, n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin, warfarin, or clopidogrel</td>
<td>781 (92)</td>
<td>771 (90)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>729 (85)</td>
<td>698 (82)</td>
</tr>
<tr>
<td>β-Blocker</td>
<td>602 (71)</td>
<td>624 (73)</td>
</tr>
<tr>
<td>Statin</td>
<td>629 (74)</td>
<td>619 (72)</td>
</tr>
<tr>
<td>ACE inhibitor or ARB</td>
<td>529 (62)</td>
<td>559 (65)</td>
</tr>
<tr>
<td>Clopogrel</td>
<td>200 (24)</td>
<td>225 (27)</td>
</tr>
<tr>
<td>Warfarin</td>
<td>60 (7)</td>
<td>88 (11)</td>
</tr>
<tr>
<td>Oral hypoglycemic diabetes medication</td>
<td>207 (25)</td>
<td>173 (21)</td>
</tr>
<tr>
<td>Insulin</td>
<td>71 (9)</td>
<td>89 (11)</td>
</tr>
<tr>
<td>Multivitamin</td>
<td>344 (42)</td>
<td>371 (45)</td>
</tr>
<tr>
<td>Other vitamins/minerals*</td>
<td>428 (52)</td>
<td>424 (51)</td>
</tr>
<tr>
<td>Herbal products</td>
<td>263 (32)</td>
<td>295 (36)</td>
</tr>
<tr>
<td><strong>Laboratory examinations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median total cholesterol level (IQR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mmol/L</td>
<td>4.25 (3.63–4.99)</td>
<td>4.29 (3.68–5.10)</td>
</tr>
<tr>
<td>mg/dL</td>
<td>164 (140–193)</td>
<td>166 (142–197)</td>
</tr>
<tr>
<td>Median triglyceride level (IQR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mmol/L</td>
<td>1.6 (1.1–2.3)</td>
<td>1.6 (1.1–2.3)</td>
</tr>
<tr>
<td>mg/dL</td>
<td>141 (99–206)</td>
<td>138 (93–202)</td>
</tr>
<tr>
<td>Median glucose level (IQR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mmol/L</td>
<td>5.6 (5.1–6.8)</td>
<td>5.7 (5.2–6.7)</td>
</tr>
<tr>
<td>mg/dL</td>
<td>102 (92–122)</td>
<td>103 (93–120)</td>
</tr>
<tr>
<td>Median LDL cholesterol level (IQR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mmol/L</td>
<td>2.28 (1.73–2.93)</td>
<td>2.30 (1.76–3.03)</td>
</tr>
<tr>
<td>mg/dL</td>
<td>88 (67–113)</td>
<td>89 (68–117)</td>
</tr>
<tr>
<td>Median HDL cholesterol level (IQR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mmol/L</td>
<td>1.11 (0.96–1.32)</td>
<td>1.09 (0.93–1.29)</td>
</tr>
<tr>
<td>mg/dL</td>
<td>43 (37–51)</td>
<td>42 (36–50)</td>
</tr>
<tr>
<td>Median serum creatinine level (IQR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>μmol/L</td>
<td>84 (79–106)</td>
<td>84 (79–106)</td>
</tr>
<tr>
<td>mg/dL</td>
<td>1.1 (0.9–1.2)</td>
<td>1.1 (0.9–1.2)</td>
</tr>
</tbody>
</table>

ACE = angiotensin-converting enzyme; ARB = angiotensin-receptor blocker; BMI = body mass index; CABB = coronary artery bypass grafting; DBP = diastolic blood pressure; HDL = high-density lipoprotein; IQR = interquartile range; LDL = low-density lipoprotein; MI = myocardial infarction; PCI = percutaneous coronary intervention; SBP = systolic blood pressure.
* Participants were receiving these agents at baseline/before initiation of the study.

Treatment Adherence

A total of 784 (46%) patients discontinued their regimen during the study (390 [46%] in the placebo group and 394 [46%] in the vitamin group; P = 0.67) (Appendix Figure, available at www.annals.org). Patients received caplets for a median of 31 months (IQR, 13 to 59 months) in the vitamin group and 35 months (IQR, 13 to 60 months) in the placebo group (P = 0.65). A total of 645 (76%) vitamin recipients and 646 (76%) placebo recipients completed at least 1 year of oral therapy (P = 0.98), and 400 (47%) and 426 (50%), respectively, completed at least 3 years (P = 0.23) (Table 1 of the Supplement, available at www.annals.org).

The most common reason for discontinuation was declining to continue taking the vitamins or placebo (74% in the vitamin group and 78% in the placebo group; P = 0.24), but 5.6% and 4.9%, respectively, discontinued because of physician preference (P = 0.65), and 9.9% and 7.9%, respectively, discontinued because of adverse events or effects (P = 0.34) (Table 2 of the Supplement). Women were more likely to discontinue vitamin therapy than men (Table 3 of the Supplement).

The only difference between groups at baseline among patients who discontinued their regimens was a higher proportion of diabetes and greater use of oral hypoglycemic drugs in the high-dose vitamin group (Table 4 of the Supplement). A total of 289 (17%) patients withdrew from the study (Figure 1); these data did not differ by group (P = 0.69).

Primary and Secondary Outcomes

The primary end point occurred in 230 (27%) patients in the vitamin group and 253 (30%) in the placebo group. The Kaplan–Meier 5-year event rate estimates were 34.2% (95% CI, 30.5% to 37.9%) for the vitamin group and 37.0% (CI, 33.2% to 40.8%) for the placebo group (hazard ratio, 0.89 [CI, 0.75 to 1.07]; P = 0.21) (Figure 2, top). Treatment comparisons of the individual components of the primary end point were indeterminate because of fewer events for each component (Table 3). The composite of cardiovascular death, MI, or stroke occurred in 94 (11%) patients in the vitamin group and 115 (13%) in the placebo group (hazard ratio, 0.82 [CI, 0.62 to 1.07]; P = 0.142) (Figure 2, bottom).

Adverse Events

Serious adverse events occurred in 124 (15%) vitamin recipients and 103 (12%) placebo recipients (difference, 3 percentage points [CI, −0.7 to 5.7 percentage points]) (Table 5 of the Supplement). Adverse events included 12 (1.4%) incident neoplasms in the vitamin group and 11 (1.3%) in the placebo group (difference, 0.1 percentage point [CI, −0.8 to 1.3 percentage points]). No evidence suggested harm from vitamin therapy in any category of adverse events (Table 6 of the Supplement).
Subgroup Analysis

Prespecified tests for treatment interactions (Figure 3) indicated no statistically significant interaction ($P = 0.94$) of oral vitamin therapy and EDTA chelation or placebo or between oral vitamin therapy and type of enrolling practice, defined as complementary or alternative medicine or conventional medical practice ($P = 0.39$). Statin therapy at baseline interacted with vitamin therapy ($P$ for interaction $= 0.012$).

Discussion

TACT found that a 28-component, high-dose oral multivitamin and multimineral regimen used as secondary prevention in patients who have had MI did not statistically significantly reduce cardiovascular events. The complex mixture seemed safe. However, these conclusions must be interpreted cautiously because of a high rate of withdrawal and nonadherence.

Our study adds to the existing literature on vitamin therapy in 3 ways. First, complementary and alternative medicine practitioners rather than clinical researchers or supplement companies designed the specific components of the oral treatment regimen, leading to a unique high-dose mixture (Table 7 of the Supplement). Second, an English-language MEDLINE search up to August 2013 showed only 1 other large-scale trial of a multivitamin preparation focusing on cardiovascular outcomes (15) that tested more than 4 components. That trial, the Physicians Health Study II, included only 5.1% ($n = 754$) of patients with self-reported vascular disease. Thus, our multivitamin study, with its multiple high-dose components and enrollment of 1708 patients who have had MI, adds to the knowledge base of multivitamin therapy as secondary prevention. Finally, and most relevant to the complementary and alternative medicine community, the $2 \times 2$ factorial design permitted the determination that vitamin therapy did not interact with intravenous chelation, an intervention that had a modestly positive effect on cardiovascular outcomes (8).

Cardiovascular disease remains the principal cause of death and disability in the United States. Among the interventions used by patients to treat or prevent heart disease are those supported by an evidence base and prescribed by physicians and over-the-counter nutritional supplements, vitamins, and minerals advertised by the vitamin and supplement industry and purchased by many patients. Clinical trials have randomly assigned thousands of patients to trials testing only a few antioxidant vitamins and minerals, typically vitamin C, vitamin E, β-carotene, and selenium, alone or in factorial groups and combinations.

The systematic analyses of trials testing a few vitamins and minerals to prevent cardiovascular disease can be characterized as negative and have suggested that some supplements may be harmful in high doses and for some patients.
Admittedly, some observations correlate diets rich in varied micronutrients to cardiovascular health and show plausible mechanisms by which complex mixtures of vitamins, minerals, and micronutrients could improve cardiovascular outcomes (1–3). Micronutrients, including vitamin C (20), some bioflavonoids, and others (21), may improve endothelial function. Vitamin E is an antioxidant vitamin and may even repair iron handling within the atherosclerotic plaque, thereby influencing oxidant damage (22). Many other mechanisms have been described that are beyond the scope of this discussion. Moreover, the safety concerns raised by clinical trials with single antioxidant vitamins have not been addressed with complex multivitamin and multimineral mixtures (4, 23, 24). Thus, it is reasonable to expand the reach of vitamin trials and test complex mixtures to elucidate efficacy and safety.

The high-dose vitamins used in TACT showed an 11% relative reduction in the primary composite end point relative to the placebo group that was not statistically significant. This difference was substantially smaller than the trial was powered to measure. Thus, although this trial does not support the routine use of this high-dose oral multivitamin regimen for all patients who have had MI, the reduced statistical power due to a small difference between groups, as well as nonadherence to the study regimen, limits the conclusion of nonefficacy. Future studies of this particular regimen would have to consider a smaller effect size than we estimated, as well as the barriers to adherence that were identified.

We found a significant interaction of vitamin therapy with statin use, reflecting a greater effect of high-dose vitamins in patients not receiving a statin. This finding, which addressed a prespecified subset of patients intolerant to statins or self-selected not to receive statins, should not be interpreted as evidence that vitamin therapy can safely be substituted for statins in patients who have had MI. This finding requires additional mechanistic research and independent replication before the clinical implications can be understood. We also did not replicate the results of Brown and colleagues (25), who observed that a reduction in clinical events associated with simvastatin was attenuated by the concomitant use of vitamins E and C, β-carotene, and selenium.

Despite our conclusions that high-dose oral vitamins and minerals alone do not seem to have a role in the management of patients who have had MI, these persons will probably continue using vitamins for cardiovascular health. It is, therefore, important to comment further on the safety of the TACT vitamins. Despite the doses used (higher in most components than those used by Sesso and associates [15]), serious adverse events and incident cases of cancer did not differ between the groups. However, this conclusion must be tempered by a high rate of discontinuation of the randomly assigned therapy or placebo.

Our study had important limitations. The statistical plan was based on an effect size (25% reduction) that may have been overly optimistic for the oral vitamins. The TACT vitamin regimen, requiring 6 large caplets daily, imposed a barrier to patient adherence.

In addition, the patient burden of the chelation component of the factorial trial was high. Combining an oral vitamin regimen with intravenous therapy probably increased the nonadherence rate for the oral therapy reported here. This nonadherence rate reduced the ability to definitively comment about the potential toxicity of such a high-dose vitamin and mineral mixture.

Nevertheless, the loss of outcomes data is at least partially mitigated because the death status of all patients was checked at the end of the study using the Social Security Death Index and the Canadian death registry. In addition, although more patients withdrew from the study than expected, some did so after having a primary end point. Patients who discontinued vitamins or placebo continued to be followed (unless they withdrew from the study); therefore, we obtained follow-up information for those who discontinued vitamins or placebo but remained in the trial.

In stable patients with a history of MI receiving appropriate, evidence-based medical therapy, use of high-
dose oral multivitamins and multiminerals seemed safe but did not statistically significantly reduce cardiovascular events. These conclusions must be interpreted cautiously because of a high rate of nonadherence to the study regimen.

From Mount Sinai Medical Center, Miami Beach, Florida; National Heart, Lung, and Blood Institute and National Center for Complementary and Alternative Medicine, Bethesda, Maryland; Palmer Center for Chiropractic Research, Davenport, Iowa; Duke Clinical Research Institute, Durham, North Carolina; Biogenesis Medical Center, Landrum, South Carolina; Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts; and University of Kansas Medical Center, Kansas City, Kansas.

**Note:** Dr. Lamas had full access to all of the data in the study and had final responsibility for the decision to submit this article for publication.

---

**Figure 3. Subgroup analyses comparing high-dose vitamins with placebo.**

<table>
<thead>
<tr>
<th>Participant Group</th>
<th>Patients/Events, n/N</th>
<th>Interaction P Value</th>
<th>Hazard Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All participants</td>
<td>853/230</td>
<td>0.89 (0.75–1.07)</td>
<td></td>
</tr>
<tr>
<td>Infusions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>421/108</td>
<td>0.89 (0.68–1.15)</td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>432/122</td>
<td>0.90 (0.70–1.15)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td>0.171</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>706/188</td>
<td>0.84 (0.69–1.03)</td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>147/42</td>
<td>1.17 (0.75–1.83)</td>
<td></td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>797/215</td>
<td>0.90 (0.75–1.09)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>56/15</td>
<td>0.82 (0.40–1.67)</td>
<td></td>
</tr>
<tr>
<td>Time from MI to enrollment</td>
<td></td>
<td>0.046</td>
<td></td>
</tr>
<tr>
<td>&lt;2 y</td>
<td>246/67</td>
<td>0.74 (0.54–1.02)</td>
<td></td>
</tr>
<tr>
<td>2–5 y</td>
<td>199/36</td>
<td>0.67 (0.44–1.02)</td>
<td></td>
</tr>
<tr>
<td>≥5 y</td>
<td>408/127</td>
<td>1.11 (0.87–1.43)</td>
<td></td>
</tr>
<tr>
<td>Anterior MI</td>
<td></td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>341/83</td>
<td>0.93 (0.69–1.26)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>512/147</td>
<td>0.88 (0.70–1.09)</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>282/85</td>
<td>0.84 (0.62–1.14)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>571/145</td>
<td>0.90 (0.72–1.12)</td>
<td></td>
</tr>
<tr>
<td>Previous revascularization</td>
<td></td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>705/180</td>
<td>0.87 (0.71–1.06)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>148/50</td>
<td>0.97 (0.66–1.45)</td>
<td></td>
</tr>
<tr>
<td>Statins at baseline</td>
<td></td>
<td>0.012</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>629/179</td>
<td>1.03 (0.84–1.27)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>224/51</td>
<td>0.62 (0.44–0.87)</td>
<td></td>
</tr>
<tr>
<td>CAM site</td>
<td></td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>544/141</td>
<td>0.84 (0.67–1.05)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>309/89</td>
<td>0.99 (0.74–1.33)</td>
<td></td>
</tr>
</tbody>
</table>

CAM = complementary and alternative medicine; MI = myocardial infarction.
Disclaimer: The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the National Heart, Lung, and Blood Institute; National Center for Complementary and Alternative Medicine; or National Institutes of Health.

Acknowledgment: The authors thank the TACT investigators (members are listed in the Appendix, available at www.annals.org). They also thank Ana Mon, MPH, Project Leader at the Clinical Coordinating Center, for her organizational skills; Alyssa Cotler at the National Center for Complementary and Alternative Medicine, Susan Dambrasuskas (formerly at the National Heart, Lung, and Blood Institute), and Vivian Thompson at the Duke Clinical Research Institute for their competent professional assistance; and the Florida Heart Research Institute for supporting the pilot study.

Grant Support: Grant U01HL092607 from the National Heart, Lung, and Blood Institute and grant U01AT001156 from the National Center for Complementary and Alternative Medicine of the National Institutes of Health.

Potential Conflicts of Interest: Disclosures can be viewed at www.acponline.org/authors/icmje/ConflictOfInterestForms.do?msNum=M13-1530.

Reproducible Research Statement: Study protocol, statistical code, and data set: Not available.

Requests for Single Reprints: Gervasio A. Lamas, MD, Columbia University Division of Cardiology, Mount Sinai Medical Center, 4300 Alton Road, Miami Beach, FL 33140; e-mail, gervasio.lamas@msmc.com.

Current author addresses and author contributions are available at www.annals.org.

References
Current Author Addresses: Dr. Lamas: Columbia University Division of Cardiology, Mount Sinai Medical Center, 4300 Alton Road, Miami Beach, FL 33140.
Drs. Boineau, Rosenberg, and Stylianou: National Heart, Lung, and Blood Institute, 6701 Rockledge Drive, MSC 7956, Bethesda, MD 20892.
Dr. Goertz: Palmer Center for Chiropractic Research, 741 Brady Street, Davenport, IA 52804.
Drs. Mark and Lee and Ms. Lindblad: Duke Clinical Research Institute, 2400 Pratt Street, Durham, NC 27705.
Dr. Rozema: Biogenesis Medical Center, 1000 East Rutherford Road, Landrum, SC 29356.
Dr. Nahin: National Center for Complementary and Alternative Medicine, 31 Center Drive, Room 2 B-11, Bethesda, MD 20892.
Dr. Lewis: Brigham and Women’s Hospital and Harvard Medical School, 75 Francis Street, Boston, MA 02115.
Dr. Drisko: Integrative Medicine, University of Kansas Medical Center, 3901 Rainbow Boulevard, MS 1017, Kansas City, KS 66160.

Provision of study materials or patients: G.A. Lamas, J. Drisko.
Obtaining of funding: G.A. Lamas, R. Boineau, D.B. Mark.
Administrative, technical, or logistic support: G.A. Lamas, R. Boineau, Y. Rosenberg, T. Rozema.
Collection and assembly of data: D.B. Mark, E.F. Lewis, J. Drisko.

APPENDIX: TACT INVESTIGATORS, LEADERSHIP, AND TRIAL COMMITTEES

United States
Bronx-Lebanon Hospital Center, Bronx, New York: Narendra Bhulodkar, Noneta Montinola; Magaziner Center for Wellness, Cherry Hill, New Jersey: Allan Magaziner, Betty Ann Persico; The Ohio State University Medical Center, Columbus, Ohio: Raymond Magorien, Luba Mazanec; The Johns Hopkins University, Baltimore, Maryland: Pamela Ouyang, Jeanne Wingo; Baystate Medical Center, Springfield, Massachusetts: Mara Slawsky, Judith Fleurent; Heart Care Center, East Syracuse, New York: Russell Silverman, Sherri Loucks; Androscoggin Cardiology Associates, Auburn, Maine: Robert Weiss, Diana Cass; Family Health Medical Services, Mayville, New York: Robert Berke, Paige Davidson; Main Line Health Heart Center, Wynnewood, Pennsylvania: Robert Bulgarelli, Susan Herring; New York Veterans Affairs Medical Center, Cardiovascular Clinical Research Center, New York, New York: Steven Sedlis, Estelita Anteola; University Hospitals of Cleveland, Cleveland, Ohio: Austin Halle, Lian Yang; The Institute of Integrative Medicine, Denville, New Jersey: Majid Ali, Mahboobullah Baig; Deborah Heart and Lung Center, Browns Mills, New Jersey: Alexander Poulathas, Linda Dewey; New York University School of Medicine, New York, New York: Harmony Reynolds, Chao Wang; Rheinebeck Health Center, Rheinebeck, New York: Kenneth Bock, Debbie Truin; Schachter Center for Complementary Medicine, Suffern, New York: Michael Schachter, Sally Minniefeld; Yulius Poplyansky, MD, Roselle Park, New Jersey: Yulius Poplyansky, Marjorie Patino; Stockton Family Practice, Stockton, New Jersey: Stuart Freedenfeld, Verna Good; Hudson Valley Heart Center, Poughkeepsie, New York: Glenn Gerber, Patricia O’Brien; Celebration of Health Association, Bluffton, Ohio: Terry Chappell, Marcia Arnold; Wholistic Health Center, Greensburg, Pennsylvania: Ralph Miranda, Barb Casella; Staten Island Heart, Staten Island, New York: James Lafferty, Lenora Tafuri-Acevedo; Marino Center for Integrative Medicine, Cambridge, Massachusetts: Guy Pugh, Vivian Cole; Land Clinical Studies, West Caldwell, New Jersey: James Garofalo, Krystle Chavez; Comprehensive Heart Care, Toledo, Ohio: James Roberts, Debra Braun; Advanced Family Medicine, Grove City, Ohio: James Johnson, Rosemary Stevenson; Longevity Medical, South Amboy, New Jersey: Ivan Krohn, Lewis S. Korb; Lake Cable Medical Center, Canton, Ohio: Jack Slingluff, John Mountford; Woodlands Healing Research, Quakertown, Pennsylvania: Robert Schmidt, Evelyn Alentin; Matrix Clinic, Dayton, Ohio: Lisa Lichota, Keith Rost; Maine Integrative Wellness, Portland, Maine: Sean McCloy; Upper Valley Family Care, Troy, Ohio: Richard Plumb, Lynn Shough; Arkansas Center for Physical Medicine and Rehabilitation, North Little Rock, Arkansas: Linda Bunch, April Archeyl; Riverside Family Medical, Lawrenceville, Georgia: Lisa Merritt, Lisa Lockett; Florida Cardiovascular Group, Atlantis, Florida: Steven Borzak, Dina Herig; University of Arkansas for Medical Sciences, Little Rock, Arkansas: Joseph Bissett, Sandra McLaren; Central Arkansas Veterans Healthcare System, Little Rock, Arkansas: Joseph Bissett, Sharon Locke; The Heart Group, Fort Myers, Florida: Joseph O’Bryan, Mary Barr; Boice Willis Clinic, Rocky Mount, North Carolina: Shalendra Varma; Cardiology Consultants of South Florida, Tamarac, Florida: Ricky Schneider, Rochelle Mckenzie; Complementary Medical Services, Mandeville, Louisiana: James Carter, Kaylynn LeBlanc; Athens Surgery Clinic, Athens, Tennessee: Joseph Holliday, Vivian Holliday; Biogenesis Medical Center, Landrum, South Carolina: Theodore Rozema, Dolly Corbin; Mount Sinai Medical Center, Miami Beach, Florida: Robert Ciccia-Maclean, Pablo Guala; Mount Sinai Medical Center of Florida, Miami Beach, Florida: Todd Heimowitz, Helen Garcia; Caring Cardiology, Miami Beach, Florida: Roy Heilbron, Celia Heilbron; Pain and Healing Center, Miami Springs, Florida: Angelique Hart; Baptist Cardiac and Vascular Institute, Miami, Florida: Barry Katzen, Ivette Cruz; Advantage Health Center, Myrtle Beach, South Carolina: Donald Tice; Wellness and Longevity Center of Louisiana, Lafayette, Louisiana: Sangeeta Shah, Debbie Vige; Virginia Beach General Hospital, Virginia Beach, Virginia: John Griffin, Pam Hollsten; Tri-Health Alternative Medical Center, Mount Dora, Florida: Jack Young, Estela Fransbergen; Tru Med, Melbourne, Florida: Rajiv Chandra, Terry
Jean-Claude Tardif, Randa Zamrini; Cline Medical Centre, Nanaimo, British Columbia: John Cline, Frank Pluta.

Data and Safety Monitoring Board
Howard Hodis (Chair), Steven Buckley, Barry R. Davis, Theodore Ganiats, Gail Geller, Robert Nash, George Wyse.

Committees and Coordinating Centers
Clinical Events Committee at the Brigham and Women’s Hospital, Boston, Massachusetts: Marc Pfeffer, Eldrin Lewis, Peter Finn, Chau Duong, Renée Mercier.

Data Coordinating Center at the Duke Clinical Research Institute, Durham, North Carolina: Kerry Lee (principal investigator), Sandra Tourt-Uhlig, Joyce Good, Lauren Lindblad, Loren Lytle, Vivian Thompson, Linda Szczech, Gerard Esposito, Meredith Smith, Trevorlyn Haddock, Constance Bardinelli, Wanda Parker, Lindsey Lambe, Cresha Cianciolo, Brian Fox, Emlie Johnson, Mary Molina, Rita Weber, Leslie Williams.

Economics and Quality of Life Coordinating Center at the Duke Clinical Research Institute, Durham, North Carolina: Daniel Mark (principal investigator), Nancy Clapp-Channing, Diane Minshall-Liu, Jason Blevins, Kevin Anstrom, David Knight, Thomas Redick, Andrea Davis, Miguel Pena.

Clinical Coordinating Center at Mount Sinai Medical Center, Miami Beach, Florida: Gervasio Lamas (principal investigator), Ana Mon, Esteban Escolar, Steven Hussein, Pablo Guala, Kayvan Amini, Faisal Shamshad, Jacqueline Arciniega, Jamie Zimmerman, Danielle Hollar, Beatriz Acevedo, Helen Garcia, Adam Williams, Matthew Shields, Renea Moss, Virginia Martini, Parminder Singh, Jewmaull Reed, Maria Salas, Carlos Zamora, Tristan Edwards, Stephanie Escalante, Laura Davila, Rachel Margolis.

Appendix Figure. Kaplan–Meier estimates of discontinuation of vitamins: high-dose vitamins versus placebo.

Hazard Ratio, 1.03 (95% CI, 0.90–1.19); P = 0.67

Patients at risk, n
High-dose vitamins 853 707 645 571 504 433 400 359 320 256 160
Placebo 855 725 646 578 515 463 426 377 332 280 176

www.annals.org
Hip bone loss is attenuated with 1000 IU but not 400 IU daily vitamin D3: a 1 year double-blind RCT in postmenopausal women. J Bone Min Res. 2013.
Hip Bone Loss Is Attenuated With 1000 IU but Not 400 IU Daily Vitamin D3: A 1-Year Double-Blind RCT in Postmenopausal Women

Helen M Macdonald,1* Adrian D Wood,1 Lorna S Aucott,1 Alison J Black,2 William D Fraser,3 Alexandra Mavroeidi,4 David M Reid,1 Karen R Secombes,1 William G Simpson,5 and Frank Thies1

1School of Medicine and Dentistry, University of Aberdeen, Aberdeen, UK
2Grampian Osteoporosis Service, Woolmanhill Hospital, Aberdeen, UK
3Norwich Medical School, University of East Anglia, Norwich, UK
4School of Medical Sciences, University of Aberdeen, Aberdeen, UK
5Aberdeen Royal Infirmary, Aberdeen, UK

ABSTRACT
Few year-long vitamin D supplementation trials exist that match seasonal changes. The aim of this study was to determine whether daily oral vitamin D3 at 400 IU or 1000 IU compared with placebo affects annual bone mineral density (BMD) change in postmenopausal women in a 1-year double-blind placebo controlled trial in Scotland. White women aged 60 to 70 years (n = 305) were randomized to one of two doses of vitamin D or placebo. All participants started simultaneously in January/February 2009, attending visits at bimonthly intervals with 265 (87%) women attending the final visit and an additional visit 1 month after treatment cessation. BMD (Lunar iDXA) and 1,25-dihydroxyvitamin D[1,25(OH)2D], N-terminal propeptide of type 1 collagen [P1NP], C-terminal telopeptide of type I collagen [CTX], and fibroblast growth factor-23 [FGF23] were measured by immunoassay at the start and end of treatment. Circulating PTH, serum Ca, and total 25-hydroxyvitamin D [25(OH)D] (latter by tandem mass spectrometry) were measured at each visit. Mean BMD loss at the hip was significantly less for the 1000 IU vitamin D group (0.05%/C6 1.46%) compared with the 400 IU vitamin D or placebo groups (0.57%/C6 1.33% and 0.60%/C6 1.67%, respectively) (p < 0.05). Mean (#/SD) baseline 25(OH)D was 33.8%/C6 14.6 nmol/L; comparative 25(OH)D change for the placebo, 400 IU, and 1000 IU vitamin D groups was –4.1%/C6 11.5 nmol/L, +31.6%/C6 19.8 nmol/L, and +42.6%/C6 18.9 nmol/L, respectively. Treatment did not change markers of bone metabolism, except for a small reduction in PTH and an increase in serum calcium (latter with 1000 IU dose only). The discordance between the incremental increase in 25(OH)D between the 400 IU and 1000 IU vitamin D and effect on BMD suggests that 25(OH)D may not accurately reflect clinical outcome, nor how much vitamin D is being stored.

© 2013 American Society for Bone and Mineral Research.

KEY WORDS: VITAMIN D; RANDOMIZED CONTROLLED TRIAL; BONE LOSS; BONE TURNOVER MARKERS; POSTMENOPAUSAL WOMEN

Introduction

Vitamin D has been synonymous with bone health since its discovery in the early 20th century.11 It has a clear role in preventing rickets and osteomalacia and in helping absorb sufficient dietary calcium necessary for mineralizing bones, but we are still unclear about the extent of its involvement in protecting against osteoporosis. A number of trials have investigated the effect of vitamin D treatment on fracture risk with mixed results, which may be explained by type of vitamin D treatment (vitamin D2 versus vitamin D3); dose (daily or less frequent); duration of treatment; and administration route (oral/injection). Subsequent meta-analyses, of which there are many, have concluded that either calcium is also required23–4 or the daily vitamin D dose needs to be higher than 800 IU.5 The overall benefit appears to be weighted by one trial in which institutionalized elderly were treated with vitamin D and calcium;6 the large, pragmatic trials involving healthier elderly, which did not show any benefit, have been criticized for lack of compliance.7,8 Observational studies have suggested a
relationship between 25-hydroxvitamin D [25(OH)D], the major circulating metabolite of vitamin D, and bone mineral density (BMD) \(^9\text{–}\text{11}\) or reduced fracture. \(^12,13\) But even then, the data are equivocal. There are some discrepancies about how much oral vitamin D is required to increase 25-hydroxvitamin D [25(OH)D] by a fixed amount. \(^14,15\) The 25(OH)D increase may be greater if the starting point is lower, and an initial high starting point of 25 (OH)D (mean 71 nmol/L) was suggested as a reason for the nonresponse of BMD to vitamin D supplementation in a recent trial. \(^16,17\) A recent dose-response study found that increasing amounts of vitamin D resulted in successively smaller incremental increases in 25(OH)D. \(^18\)

Although the starting 25(OH)D concentration may be critical in determining the study outcome, for many studies that have investigated the role of vitamin D on bone health, there is confounding from the underlying effects of season, as 25 (OH)D is higher in summer and lower in winter. The relationship between change in vitamin D status and bone status in healthier older adults remains unclear. Few studies have included estimates of dietary intakes and sunlight exposure in their adjustments.

The aim of this study was to test whether vitamin D\(_3\) supplementation at a daily dose of 400 IU (10 \(\mu\)g) or 1000 IU (25 \(\mu\)g) for 1 year starting in January/February affects bone mineral density loss in women aged 60 to 70 years living in northeast Scotland, a population with low circulating 25(OH)D. Secondary outcomes included markers of vitamin D and bone metabolism, including total 25-hydroxyvitamin D [25(OH)D], parathyroid hormone (PTH), 1,25 dihydroxyvitamin D [\(1,25 (\text{OH})_2\text{D}\)], serum calcium, fibroblast growth hormone factor 23 (FGF23), N-terminal propeptide of type 1 collagen (P1NP), and C-terminal telopeptide of type I collagen (CTX).

Materials and Methods

Subjects

The women were healthy postmenopausal, nonsmoking women living in northeast Scotland aged 60 to 70 years who were taking part in a vitamin D intervention study (ViTaMin D and CardiOvascular Risk, VICTORy) to investigate risk of cardiovascular disease (CVD). \(^19\) At the time of recruitment, they were not suffering from any condition (diabetes, asthma, malabsorption, blood pressure > 160 mm Hg systolic or > 99 mm Hg diastolic) or taking medication (hypotensive, hypolipemic, anti-inflammatory, oral corticosteroid) likely to affect vitamin D metabolism or CVD risk. Women on thyroxine treatment were included if stable, as assessed by free T4 and thyroid stimulating hormone concentrations, and their dose had not changed in the 3 months before study entry. Exclusion criteria were planned frequent trips or long periods abroad that would result in an increased exposure to UVB light, or an abnormal biochemical profile on screening. All women provided written informed consent. Ethical permission was obtained from Grampian Research Ethics Committee (08/S0802/73). There were no differences in characteristics between the women who were analyzed for the VICTORy study \(^19\) and the women who had dual-energy X-ray absorptiometry (DXA) scans and could be included in determining the effect of the treatment on bone outcomes (data not shown). Details of the numbers recruited are included in Fig. 1.

Randomization and intervention

Capsules containing vitamin D (400 IU and 1000 IU) and identical placebo capsules were manufactured by Pure Encapsulations (Sudbury, MA, USA) and sent directly to Bilcare (Crickhowell Powys, UK), where they were packed and coded. The certificate of analysis from the manufacturer showed that each capsule contained 400 IU and 1040 IU of vitamin D. Independent analysis (by Eurofins Laboratories Ltd, Wolverhampton, UK) at the end of the study gave a vitamin D content of 346 IU and 832 IU, respectively, which they accepted as being within their quality-control specifications. Participants were randomized in January to March 2009 by the Health Services Research Unit, University of Aberdeen (telephone service) to one of three groups: placebo, 400 IU (10 \(\mu\)g) vitamin D\(_3\), or 1000 IU (25 \(\mu\)g) vitamin D\(_3\), using minimization criteria for body mass index (BMI; < 18.5, 18.5 to 24.99, 25 to 29.99, 30 to 30.99, or > 40). At each visit, the participants were given a bottle of capsules \((n = 65)\), sufficient to last 2 months, and instructed to take one capsule at the same time each day with food. Compliance was estimated by counting the unused capsules at each subsequent visit (and supported by later 25(OH)D measurements). The investigators remained blinded throughout the study until after the statistical analysis of the outcomes had been performed. This was achieved by ensuring that the 25(OH)D data was analyzed by a separate researcher (HMM) using a different set of codes from the researcher carrying out the analysis of the main outcome measures (ADW).

Anthropometric measurements

Women were weighed (Seca, Hamburg, Germany) and their height measured using a stadiometer (Holtain Ltd, Crymych, UK) at baseline, 6 months, and 12 months. They had DXA scans to estimate BMD at the hip (total) and spine (L\(_1\) to L\(_4\)), total bone mass, total lean mass, and total fat mass (Lunar iDXA, GE Medical Systems Inc., Madison, WI, USA) before the intervention started and at the end of the study. Daily phantom measurements were performed. In vivo precision was obtained using repeat scans from 60 volunteers and was 0.54% for spine (L\(_2\) to L\(_4\)) BMD and 0.56% for mean total hip BMD (left hip only 0.68%; right hip only 0.75%).

Markers of vitamin D status and bone health

Overnight fasted serum and plasma samples collected at each visit were stored at \(-80\)°C, with each participant’s complete set batched together before analysis. Measurements for 25(OH)D, PTH, and FGF23 were undertaken under the direction of WDF in the Department of Clinical Chemistry, University of Liverpool, Liverpool, UK, which takes part in the quality-control group for vitamin D, DEQAS. Serum was analyzed for 25(OH)D\(_2\) and 25(OH)D\(_3\) using dual tandem mass spectrometry using the NIST (National Institute of Standards and Technology, US) standard that is recommended. \(^20\) Interassay coefficients of variation for the assay were < 10% for both 25(OH)D\(_2\) and 25(OH)D\(_3\). The sum
of the two are reported as total 25(OH)D. The half-life of circulating 25(OH)D was calculated using the following formula:

\[
\text{half life} = \frac{\text{time since treatment ceased}}{C_2 \ln(2)} \left( \frac{\ln(25(OH)D \text{ at end of treatment})}{25(OH)D \text{ after the period of treatment cessation}} \right).
\]

Parathyroid hormone (PTH) was measured in plasma samples using an electrochemiluminescent immunoassay (ECLIA) on a Modular Analytics E170 analyzer (Roche Diagnostics, Burgess Hill, UK). Inter/intra-assay coefficient of variation was <4% from 1 to 30 pmol/L. The assay sensitivity (replicates of the zero standard) was 0.8 pmol/L. Serum 1,25(OH)_2D was measured by radioimmunoassay with a 125I-labelled 1,25(OH)_2D derivative tracer and Sac-cell separation after immunoextraction of 1,25(OH)_2D using a mini column containing a solid-phase monoclonal antibody (Immunodiagnostic Systems, Boldon, Tyne and Wear, UK). Inter/intra-assay coefficient of variation was <10% over the concentrations analyzed.\(^{(21)}\) FGF23 was measured by ELISA with an anti-human FGF-23 mouse monoclonal antibody (Kainos Laboratories, Tokyo, Japan). It has quantification range of 3 to 800 pg/mL.

Serum calcium adjusted for albumin was measured by Clinical Biochemistry, Aberdeen Royal Infirmary, UK, using standard automated systems (ADIVA 2400 Chemistry System, Siemens, Surrey, UK). Inter/intra-assay coefficient of variations over the concentrations analyzed were <2.2% for calcium and <1.8% for albumin. Serum N-terminal propeptide of type 1 collagen (P1NP) and plasma C-terminal telopeptide of type I collagen (CTX) were measured at the start and end visits only by enzyme chemiluminescent immunoassay (ECLIA) (Roche Products Ltd, Penzberg, Germany) using a Diagnostics Elecsys 2010 Immunoassay System (Roche Diagnostics, Mannheim, Germany or Burgess Hill, UK) (coefficient of variation was <4% for P1NP and <5% for CTX).

Assessment of dietary vitamin D intake, sunlight exposure and physical activity

Dietary vitamin D was assessed by the Scottish Collaborative Group food frequency questionnaire (FFQ) (http://www.foodfrequency.org) that was completed after each visit; sunlight

---

Fig. 1. Participant flow through the study. Fewer measurements are available at the hip because of total hip replacements.
exposure was determined by polysulphide badges. These were worn at the lapel on outside clothing for the week after the study visit and returned by post. The difference in absorption at 330 nm before and after the badge had been worn, $\Delta A_{330}$, was used to obtain the weekly standard erythema dose (SED) as follows: $\text{SED} = 10.7 [\Delta A_{330}] + 14.3 [\Delta A_{330}]^2 - 26.4 [\Delta A_{330}]^3 + 89.1 [\Delta A_{330}]^4$ (Perkin Elmer UV/VIS Lambda 2 Spectrophotometer). At each visit, the women were questioned about time spent outside and how much of the body was uncovered (5% face only to 60% for face, hands, and arms or legs, plus trunk). The women were questioned about any holidays taken in the previous 2 months. Physical activity was estimated using a bone-specific questionnaire that has been validated in this age group.

Energy expended (MET hours per week) and the magnitude of the mechanical load on the skeleton were estimated using the responses to 29 questions about work- and home-related activities and leisure-time pursuits.

**Statistical analysis**

Using data from a previous 2-year intervention trial of women aged 55 to 65 years, where the annual percentage bone loss was $-1.02 \pm 1.61 \%$ at the lumbar spine (LS) and $-0.82 \pm 1.12 \%$ at the hip, we estimated that with 75 subjects in each group (with 100 allowing a 25% dropout as originally powered on markers of cardiovascular risk), a mean LS BMD loss of 0.75% for the placebo group would enable us to detect a significant benefit (90% power, $p = 0.05$) in the treatment group, if BMD increased by 0.11%. At 80% power, the detectable difference would be significant if the treatment group lost 0.01% BMD or less. Similarly, for total hip BMD, assuming a loss of 0.65% in the placebo group, we would have sufficient power to detect a significant treatment effect ($p = 0.05$) if the treatment group lost 0.05% or 0.13% BMD (90% and 80% power, respectively).

Statistical analysis was carried out using PASW Statistics 18 (release 18.0.2) (IBM SPSS Statistics, http://www.spss.com/software/statistics/academic/). One-way ANOVA was used to analyze the BMD change between the treatment groups with Dunnett post hoc ANOVA test to test the difference between groups. A General Linear Model was used to allow adjustment for major confounders (dietary calcium intake, physical activity). Repeated measures analysis of variance (ANOVA) with treatment $\times$ time interactions tested using Pillai’s Trace for 25(OH)D and markers of bone turnover. Analysis was carried out on an “intention to treat” basis. The per protocol analysis excluded 4 women who withdrew but continued with the study visits and $>80\%$ compliance (total $n = 255$).

**Results**

There were no differences in mean subject characteristics between the treatment groups (Table 1). Overall dietary vitamin D intake was low. Dietary calcium intakes were adequate. There were no differences in mean baseline BMD between the women who took part in the study and those who did not meet the study criteria, nor compared with the women who did not have a final bone scan (data not shown). There were no serious adverse events related to the treatment. One woman (who withdrew from the study) had elevated calcium thought to be a result of unmasking mild primary hyperparathyroidism, which was later monitored by her general practitioner. She had been on the 1000 IU daily vitamin D dose. There were 7 fractures during the study (wrist/lower arm [n = 3], foot/ankle [n = 3], and clavicle [n = 1]), with one woman having two fractures on two separate occasions. These are reported as adverse events, as the study was not powered to examine fractures. Three of the women were on placebo and three on the 400 IU vitamin D dose.

The capsule count indicated mean overall compliance of 92% (range 72% to 98%), which did not differ significantly between the three groups. There were no adverse events reported during the course of the study that were attributable to the treatment. Total 25(OH)D increased and remained high for the vitamin D-treated groups. For the placebo group, there was an increase in 25(OH)D during summer, which reflected sun exposure in the spring/summer and then a decrease during winter (Fig. 2). Sunlight exposure was positively skewed. Higher erythemal doses (SED) were received in summer compared with winter, but no differences were seen between treatment groups. Physical activity also changed throughout the study, increasing in summer and then decreasing, whereas dietary intakes of vitamin D, calcium, and dietary energy intake remained the same throughout the year (data not shown).

There was no change in CTX, P1NP, FGF23 or 1,25(OH)2D as a result of treatment (Table 2). There was a significant difference in PTH, which was observed before and after treatment (Table 2), and throughout the year as shown by repeated measures analysis (Fig. 3). At the end of treatment, PTH had decreased from baseline for both vitamin D treatment groups compared with placebo ($p < 0.001$ and $p = 0.031$ for the comparison with 1000 IU vitamin D and 400 IU vitamin D, respectively). There were small changes in adjusted serum calcium ($-0.016\text{mmol/L}$, $-0.009\text{mmol/L}$, and $0.005\text{mmol/L}$ for placebo, 400 IU vitamin D, and 1000 IU vitamin D, respectively), with the difference significant only between placebo and 1000 IU vitamin D ($p = 0.027$).

Bone loss at the hip was significantly greater for the placebo and 400 IU vitamin D groups (losing BMD at a mean rate of 0.6% and 0.6%, respectively) compared with the 1000 IU vitamin D group, which essentially showed no change ($-0.05\%$) (ANOVA post hoc comparison with 1000 IU vitamin D treatment: $p = 0.027$ for placebo; $p = 0.043$ for 400 IU vitamin D). The BMD change at the lumbar spine ($L_1$ to $L_4$) was not significantly different between the treatment groups ($-0.5\%$, $-0.2\%$, and 0.2% for placebo, 400 IU vitamin D, and 1000 IU vitamin D, respectively). Adjustment for confounders (including dietary calcium, sunlight exposure, or physical activity) did not change the outcome, nor did repeating the analysis excluding women who stopped treatment but continued study visits and those with <80% compliance. Body weight, change in CTX, change in PTH, and change in 1,25(OH)2D, but not changes in serum calcium, P1NP, and FGF23, were found to be additional independent predictors of percentage hip BMD change by ANOVA (Table 3).

There were few women with 25(OH)D >75 nmol/L at baseline ($n = 6$ total: 3 from the placebo and 3 from the 1000 IU vitamin D group) and an additional 29 in total (placebo $n = 14$; 400 IU vitamin D $n = 9$; 1000 IU vitamin D $n = 6$) with 25(OH)D >50 nmol/L. There were no differences between groups.
The differences between treatment groups for mean percentage hip BMD change was still significant if these women were excluded.

The relationship between bone markers (Table 4) showed that at baseline (i) 25(OH)D was inversely associated with PTH; (ii) serum Ca was inversely associated with the bone markers CTX and P1NP; (iii) there were inverse associations between the bone turnover markers and BMD; and (iv) 1,25(OH)2D was inversely associated with FGF23 and BMD. At the end of the treatment period, the associations were similar except in addition (i) 25(OH)D was now also positively associated with 1,25(OH)2D; (ii) serum Ca was inversely associated with CTX and no longer with P1NP; and (iii) 1,25(OH)2D was only negatively associated with LS BMD, not hip BMD or FGF23.

One month after treatment cessation, mean 25(OH)D had decreased for the two vitamin D groups but not for the placebo group. There was a large variation in the response to treatment cessation and the calculated half-life in relation to 25(OH)D decay (ie, 25(OH)D decrease occurring 1 month after treatment cessation). The number below the key cutoffs for 25(OH)D (<25 nmol/L; <50 nmol/L, and <75 nmol/L) showed significant differences between the treatment groups (Table 5).

One variable log transformed if required (dietary Ca vitamin D).

2Appendicular refers to the sum of the measurements for legs and arms.

### Table 1. Subject Characteristics at Baseline

<table>
<thead>
<tr>
<th>Subject characteristics</th>
<th>Placebo</th>
<th>400 IU</th>
<th>1000 IU</th>
<th>p^1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean ± SD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>n = 90</td>
<td>n = 84</td>
<td>n = 90</td>
<td>ANOVA</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>64.6 ± 2.3</td>
<td>64.2 ± 1.9</td>
<td>64.9 ± 2.2</td>
<td>0.121</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>69.9 ± 12.2</td>
<td>68.1 ± 11.4</td>
<td>69.4 ± 11.6</td>
<td>0.570</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>161.4 ± 6.2</td>
<td>160.3 ± 6.8</td>
<td>160.9 ± 5.2</td>
<td>0.463</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>86.5 ± 11.4</td>
<td>85.6 ± 10.1</td>
<td>87.0 ± 11.5</td>
<td>0.693</td>
</tr>
<tr>
<td>Fat mass appendicular² (kg)</td>
<td>25.9 ± 3.8</td>
<td>25.3 ± 3.9</td>
<td>25.2 ± 3.4</td>
<td>0.327</td>
</tr>
<tr>
<td>Fat mass trunk (kg)</td>
<td>12.8 ± 3.8</td>
<td>12.4 ± 3.4</td>
<td>12.6 ± 3.3</td>
<td>0.738</td>
</tr>
<tr>
<td>Lean mass appendicular² (kg)</td>
<td>14.5 ± 4.9</td>
<td>14.2 ± 4.5</td>
<td>14.5 ± 5.1</td>
<td>0.944</td>
</tr>
<tr>
<td>Lean mass trunk (kg)</td>
<td>16.6 ± 2.3</td>
<td>16.0 ± 2.4</td>
<td>16.5 ± 2.4</td>
<td>0.213</td>
</tr>
<tr>
<td><strong>Dietary calcium intake (mg/d)</strong></td>
<td>n = 90</td>
<td>n = 83</td>
<td>n = 88</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1281 ± 490</td>
<td>1253 ± 484</td>
<td>1305 ± 566</td>
<td>0.763</td>
</tr>
<tr>
<td>Dietary calcium intake with</td>
<td>n = 90</td>
<td>n = 83</td>
<td>n = 88</td>
<td></td>
</tr>
<tr>
<td>supplements (mg/d)</td>
<td>1291 ± 492</td>
<td>1261 ± 488</td>
<td>1306 ± 568</td>
<td>0.846</td>
</tr>
<tr>
<td>Dietary vitamin D (µg/d)</td>
<td>5.6 ± 3.0</td>
<td>4.6 ± 2.5</td>
<td>5.3 ± 2.9</td>
<td>0.081</td>
</tr>
<tr>
<td>Energy intake (MJ/d)</td>
<td>9.5 ± 3.0</td>
<td>8.9 ± 2.6</td>
<td>9.4 ± 3.0</td>
<td>0.412</td>
</tr>
<tr>
<td>Energy intake to basal metabolic rate ratio</td>
<td>1.71 ± 0.58</td>
<td>1.63 ± 0.49</td>
<td>1.68 ± 0.55</td>
<td>0.565</td>
</tr>
<tr>
<td><strong>Physical activity (MET h/week)</strong></td>
<td>n = 90</td>
<td>n = 81</td>
<td>n = 86</td>
<td></td>
</tr>
<tr>
<td></td>
<td>71.5 ± 31.3</td>
<td>74.7 ± 29.7</td>
<td>77.1 ± 37.3</td>
<td>0.628</td>
</tr>
<tr>
<td>Mechanical component of physical activity (peak score)</td>
<td>4.6 ± 1.9</td>
<td>4.6 ± 1.8</td>
<td>5.0 ± 1.7</td>
<td>0.353</td>
</tr>
<tr>
<td><strong>Median sunlight exposure (SED)</strong> baseline</td>
<td>n = 90</td>
<td>n = 83</td>
<td>n = 88</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.524</td>
</tr>
<tr>
<td>Median sunlight exposure (SED) v3 summer</td>
<td>4.7</td>
<td>3.9</td>
<td>5.4</td>
<td>0.395</td>
</tr>
</tbody>
</table>

SED = standard erythemal dose.

---

**Discussion**

This study showed that the group supplemented with 1000 IU vitamin D a day showed negligible mean hip BMD losses of 0.05% after 1 year of treatment compared with losses of 0.6% for the...
placebo and the 400 IU vitamin D groups, the latter bone loss being similar to the assumption for our a priori power calculation (0.65%). We found no differences in LS BMD loss between the three groups, although the overall bone loss at this site was much lower than that we predicted (mean bone loss for the placebo group was 0.5% and we expected this to be 0.75%). In this age group, LS BMD loss of some women may be masked by osteophyte growth or possibly fractures that may result in artefactually increased BMD in the region of the collapsed vertebra.

Our findings are in agreement with the conclusions that more than 400 IU a day may be required to improve bone health and that the mean 25(OH)D concentration at which benefit occurs is >74 nmol/L (mean 25(OH)D reached in our study was 76 nmol/L for the 1000 IU treatment). When we excluded women with baseline 25(OH)D >50 nmol/L, which is the threshold above which the Food and Nutrition Board of the Institute of Medicine (IOM) concluded that 97.5% of the population would be vitamin D replete, our findings did not change. Their recommendations to meet this are 600 IU vitamin D a day for adults. A recent 1-year intervention study in women aged 50 to 80 years compared two doses of vitamin D (daily 800 IU and 6500 IU, the latter as 20,000 IU twice weekly in addition to 800 IU daily) and found no differences in BMD change (no change from baseline for LS and a small increase in BMD for the hip) between the groups. However, they did not have a placebo comparison. Both groups started with much higher circulating 25(OH)D than our study (71 nmol/L).

The increase in 25(OH)D per unit of ingested vitamin D in our study was greater than others have reported, but it is known that the 25(OH)D response to vitamin D supplementation is greater if the starting 25(OH)D is lower. However, it is unclear why a small additional increase in 25(OH)D would result in reduced hip BMD loss. There was a marked increase in 25(OH)D observed for the 400 IU vitamin D group compared with the placebo with only a small additional increment in 25(OH)D for the 1000 IU vitamin D group (in spite of the latter being two and a half times the dose). Even for the summer visit, when serum 25(OH) D was at its peak for the placebo and there was a plateau for the treatment groups, the change from baseline was twice that for the 400 IU compared with the placebo, but the 1000 IU dose produced only a 20% additional increment in 25(OH)D above that for the 400 IU dose. The diminishing returns of increased vitamin D supplementation on 25(OH)D response has been noted by others. One could speculate that the additional vitamin D in the higher-dose group was not completely converted to 25(OH)D and the excess may have been deposited into tissue stores or perhaps degraded by the body and excreted. It is also possible that less vitamin D was absorbed from the gastrointestinal tract for the 1000 IU dose compared with the 400 IU dose. Discrepancies could arise because of differences in the vitamin D content of the capsules (perhaps because of different degradation rates). However, independent analysis after the study finished showed that although the high-dose capsule contained 16.8% less than the target amount and the low dose 13.5% less, the vitamin D content was still within the analytical specifications and the ratio of the two doses at the two time points was almost identical (the final ratio being 2.4 instead of the expected 2.5). We observed a small increase in serum calcium for the 1000 IU vitamin D treatment group, and it is possible that transient increases in serum calcium after ingestion of the higher-dose vitamin D contribute to a bone anabolic effect (although analysis of serum P1NP, a bone formation marker, showed no change). Alternatively, our data may reflect increased mineralization of undermineralized bone. The magnitude of change for serum calcium was very small, with the final mean concentration being the same for all three groups, but the differences from baseline between treatment groups were statistically significant. Alternatively, the degradation of excess vitamin D could stimulate metabolic pathways that might be beneficial to bone, although this is largely speculative. Other metabolites of vitamin D, produced as a result of 24-hydroxylation, for example, may be involved when higher vitamin D doses are ingested. Although it is possible that additional vitamin D improved muscle strength and increased physical activity resulted in improved BMD, our study was not designed to test this.

We did not observe any between-group differences in circulating bone turnover markers (final or change from baseline) that could help explain the mechanism behind our findings (although CTX, 1,25(OH)2D, and PTH were independent predictors of change in BMD). The only marker for which there was a difference between groups was PTH. Although there appeared to be a dose response with the reduction in PTH for the 1000 IU vitamin D treatment being greater than that for the 400 IU, the final circulating PTH for the two vitamin D–treated groups was identical. The inclusion of FGF23 in the analysis did not influence the outcomes. This growth factor is important in regulating
### Table 2. Changes in Markers of Vitamin D Status and Bone Health

<table>
<thead>
<tr>
<th></th>
<th>Baseline (visit 0)</th>
<th>Final (visit 6)</th>
<th>Difference</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo 400 IU</td>
<td>Placebo 1000 IU</td>
<td>Placebo</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>n = 90</td>
<td>n = 84</td>
<td>n = 90</td>
<td></td>
</tr>
<tr>
<td>25(OH)D (nmol/L)</td>
<td>35.8 ± 16.4</td>
<td>33.4 ± 13.2</td>
<td>33.6 ± 13.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>400 IU</td>
<td>1000 IU</td>
<td>p</td>
<td></td>
</tr>
<tr>
<td>1,25(OH)2D (pmol/L)</td>
<td>143 ± 43</td>
<td>138 ± 40</td>
<td>138 ± 46</td>
<td></td>
</tr>
<tr>
<td>PTH (pmol/L)</td>
<td>5.3 ± 1.3</td>
<td>4.8 ± 1.3</td>
<td>5.1 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Serum Ca (mmol/L)</td>
<td>2.35 ± 0.07</td>
<td>2.33 ± 0.07</td>
<td>2.32 ± 0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 84</td>
<td>n = 77</td>
<td>n = 82</td>
<td></td>
</tr>
<tr>
<td>P1NP (mg/L)</td>
<td>45.1 ± 23.0</td>
<td>45.7 ± 20.2</td>
<td>45.8 ± 18.6</td>
<td></td>
</tr>
<tr>
<td>CTX (mg/L)</td>
<td>0.38 ± 0.17</td>
<td>0.41 ± 0.15</td>
<td>0.37 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>FGF23 (ng/L)</td>
<td>71.9 ± 40.0</td>
<td>83.9 ± 73.5</td>
<td>74.8 ± 67.6</td>
<td></td>
</tr>
<tr>
<td>Mean total hip BMD (g/cm²)</td>
<td>0.920 ± 0.118</td>
<td>0.917 ± 0.102</td>
<td>0.923 ± 0.132</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 90</td>
<td>n = 84</td>
<td>n = 90</td>
<td></td>
</tr>
<tr>
<td>Lumbar spine (L1 to L4)</td>
<td>1.081 ± 0.153</td>
<td>1.075 ± 0.141</td>
<td>1.068 ± 0.161</td>
<td></td>
</tr>
<tr>
<td>BMD (g/cm²)</td>
<td>n = 84</td>
<td>n = 84</td>
<td>n = 90</td>
<td></td>
</tr>
</tbody>
</table>

25(OH)D = total 25-hydroxyvitamin D; 1,25(OH)²D = total 1,25-dihydroxyvitamin D; PTH = parathyroid hormone; CTX = plasma beta C-terminal telopeptide; P1NP = serum N-terminal propeptide of type 1 collagen; BMD = bone mineral density.

ANOVA *p < 0.05; †p < 0.01; ‡p < 0.001 (variable log transformed if required).

Dunnett post hoc ANOVA tests as follows: 25(OH)D difference p < 0.001 for all comparison groups; PTH difference between placebo and 1000 IU vitamin D, p < 0.001; between 400 IU vitamin D and 1000 IU vitamin D, p = 0.031; and between placebo and 400 IU vitamin D, p = 0.071; adjusted serum calcium difference between placebo and 1000 IU, p < 0.027; percentage change in hip BMD difference between placebo and 1000 IU vitamin D, p = 0.027; and between 400 IU vitamin D and 1000 IU vitamin D, p = 0.043. For all other comparisons, p > 0.10.

CTX and P1NP had one extreme outlier that was removed for that analysis only. Similarly, FGF23 had a different extreme outlier that was removed. Including the outlier did not change the outcome.
circulating phosphate. It is known that FGF23 increases with severity of chronic kidney disease, but manipulating dietary phosphate in healthy individuals does not appear to affect FGF23, as shown when six healthy males were given oral phosphate binders in combination with low dietary phosphate intake for 2 days followed by 3 days of repletion with inorganic phosphate.\(^\text{32}\) In contrast, a study with 10 healthy subjects was able to induce changes in FGF23 (and 1,25(OH)\(_2\)D) by varying dietary phosphate and calcium.\(^\text{33}\) Our population was relatively healthy and this marker may be more meaningful in those with declining kidney function.

Vitamin D binding protein (VDBP) may modulate the activity of vitamin D metabolites, which could explain the discrepancies seen between different studies examining the relationship between 25(OH)D and bone health.\(^\text{34}\) It has also been speculated that the VDBP binding of 25(OH)D may protect 25(OH)D against degradation, which provides a mechanism by which the decrease in 25(OH)D in winter at high latitudes could be minimized. Unfortunately, we did not have VDBP measurements to test this hypothesis. Measurements of half-life of 25(OH)D range from a few weeks\(^\text{35}\) to 2 months\(^\text{36}\) but the “vitamin D winter,” the period when vitamin D cannot be synthesized, can last 6 months or more at high latitude. We found a wide variation in the calculated half-life for 25(OH)D, with a median of around 2 months for both treatment groups, which is at the upper end of published half-lives for 25(OH)D.\(^\text{37}\) It was notable that the percentage of women with 25(OH)D below 25 nmol/L 1 month after treatment stopped was essentially zero for the treatment groups compared with 40% who had no vitamin D treatment. For the 50 nmol/L cutoff, the figure given by the IOM as covering most of the population’s vitamin D needs\(^\text{28}\) showed that 16% and 50% of women were below this for 1000 IU and 400 IU treatment groups, respectively, compared with 94% for the placebo. If a higher cutoff of 75 nmol/L is considered optimal, even for the 1000 IU vitamin D dose, the number of women below this cutoff increased rapidly from 48% to 83% within 1 month. Although this indicates that the treatment should be continued to sustain 25(OH)D at high circulating concentrations, we do not know if treatment has longer-term benefits in keeping people above the lower threshold for risk of deficiency. Further, the assumption is that 25(OH)D is reflecting vitamin D stores in the body.

Our findings and those of others raise the issue of whether there is some resistance to the body achieving higher 25(OH)D with oral vitamin D. This area is one of controversy, with calls for higher circulating 25(OH)D because these concentrations are observed in outdoor workers such as lifeguards.\(^\text{38,39}\) There are contrasting data to show that less than half of those with an outdoor lifestyle, living in an area where UVB radiation is sufficient all year, could reach 75 nmol/L 25(OH)D,\(^\text{40}\) although some have argued that this is because use of sunscreen would have blocked vitamin D synthesis. It is possible that the breakdown of excess vitamin D synthesis. It is possible that the breakdown of excess vitamin D, which normally occurs to

---

**Table 3. Predictors of Percentage Hip BMD Change by Analysis of Covariance**

<table>
<thead>
<tr>
<th>Variables included in the model</th>
<th>Beta</th>
<th>SEM</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-1.779</td>
<td>0.581</td>
<td>0.002</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>0.018</td>
<td>0.008</td>
<td>0.029</td>
</tr>
<tr>
<td>1,25(OH)(_2)D difference</td>
<td>-0.006</td>
<td>0.002</td>
<td>0.013</td>
</tr>
<tr>
<td>PTH difference</td>
<td>0.243</td>
<td>0.116</td>
<td>0.037</td>
</tr>
<tr>
<td>CTX difference</td>
<td>-2.344</td>
<td>0.992</td>
<td>0.019</td>
</tr>
<tr>
<td>Treatment group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>400 IU vitamin D(^1)</td>
<td>0.250</td>
<td>0.243</td>
<td>0.305</td>
</tr>
<tr>
<td>1000 IU vitamin D(^1)</td>
<td>0.701</td>
<td>0.243</td>
<td>0.004</td>
</tr>
</tbody>
</table>

---

\(^1\)Placebo [reference].

---

**Journal of Bone and Mineral Research**

VITAMIN D RCT ON BONE 2209

---

\(^2\)Placebo [reference].

---

\(^3\)Placebo [reference].
regulate cutaneous vitamin D synthesis, is compromised in UVR-overexposed skin and this could explain high circulating concentrations of 25(OH)D in some individuals. If one accepts that sunlight can increase 25(OH)D to circulating concentrations in excess of 100 nmol/L in everyone, the dose-response rate for oral vitamin D is puzzling because it indicates that many-fold increases in vitamin D intake are required to raise mean 25(OH)D by relatively small additional increments. There are studies underway using daily doses of 4000 IU and 6000 IU that are below the no observed adverse effect level of 10,000 IU but above safe upper limit of 4000 IU suggested by IOM, but it would appear that perhaps not all of this is converted to circulating 25(OH)D. If the storage capacity of vitamin D is limited as has been suggested, resolving what happens to the excess vitamin D may appear that perhaps not all of this is converted to circulating 25(OH)D.

Table 4. Associations Between Markers of Bone Health at Baseline (Spearman Correlations)

<table>
<thead>
<tr>
<th>Baseline (visit 0) correlations</th>
<th>25(OH)D (nmol/L)</th>
<th>1,25(OH)2D (pmol/L)</th>
<th>PTH (pmol/L)</th>
<th>Serum Ca (mmol/L)</th>
<th>P1NP (mg/L)</th>
<th>CTX (mg/L)</th>
<th>FGF23 (ng/L)</th>
<th>Mean total hip BMD (g/cm²)</th>
<th>Lumbar spine BMD (g/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25(OH)D (nmol/L)</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 263</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,25(OH)2D (pmol/L)</td>
<td>0.085</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 263</td>
<td>n = 264</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTH (pmol/L)</td>
<td>−0.318†</td>
<td>0.082</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 259</td>
<td>n = 259</td>
<td>n = 259</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum Ca (mmol/L)</td>
<td>0.053</td>
<td>−0.020</td>
<td>−0.063</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 263</td>
<td>n = 263</td>
<td>n = 263</td>
<td>n = 263</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1NP (mg/L)</td>
<td>0.026</td>
<td>0.073</td>
<td>−0.019</td>
<td>0.160†</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 243</td>
<td>n = 243</td>
<td>n = 243</td>
<td>n = 243</td>
<td>n = 243</td>
<td>n = 243</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTX (mg/L)</td>
<td>0.012</td>
<td>0.081</td>
<td>0.037</td>
<td>0.168†</td>
<td>0.778†</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 241</td>
<td>n = 241</td>
<td>n = 239</td>
<td>n = 241</td>
<td>n = 241</td>
<td>n = 241</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF23 (ng/L)</td>
<td>0.021</td>
<td>−0.143</td>
<td>0.092</td>
<td>0.061</td>
<td>−0.016</td>
<td>−0.063</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 192</td>
<td>n = 192</td>
<td>n = 192</td>
<td>n = 192</td>
<td>n = 182</td>
<td>n = 181</td>
<td>n = 192</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lumbar spine BMD (g/cm²)</td>
<td>0.089</td>
<td>−0.138</td>
<td>−0.147</td>
<td>0.021</td>
<td>−0.167†</td>
<td>−0.243‡</td>
<td>0.096</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>n = 263</td>
<td>n = 264</td>
<td>n = 259</td>
<td>n = 263</td>
<td>n = 243</td>
<td>n = 241</td>
<td>n = 192</td>
<td>n = 264</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean total hip BMD (g/cm²)</td>
<td>−0.003</td>
<td>−0.157</td>
<td>−0.061</td>
<td>−0.084</td>
<td>−0.223†</td>
<td>−0.319‡</td>
<td>0.108</td>
<td>0.624†</td>
<td>1.00</td>
</tr>
<tr>
<td>n = 258</td>
<td>n = 259</td>
<td>n = 254</td>
<td>n = 258</td>
<td>n = 258</td>
<td>n = 239</td>
<td>n = 190</td>
<td>n = 259</td>
<td>n = 259</td>
<td></td>
</tr>
</tbody>
</table>

25(OH)D = total 25-hydroxyvitamin D; 1,25(OH)2D = total 1,25-dihydroxyvitamin D; PTH = parathyroid hormone; CTX = plasma beta C-terminal telopeptide; P1NP = serum N-terminal propeptide of type 1 collagen; BMD = bone mineral density.

* p < 0.05;
† p < 0.01;
‡ p < 0.001 (variable log transformed if required).
be important in determining the optimal dose for benefit. Although risk of hypercalcemia is extremely small, there may be other adverse outcomes that are affected by higher-dose vitamin D. There are already studies that suggest that high loading doses of vitamin D may be inadvisable.  

The limitations of our study include the recruitment being restricted to postmenopausal women, who were healthy but with low circulating 25(OH)D, so that the findings may not be applicable to other populations; the duration of the study being 1 year only; and that the end points were surrogate markers of bone health (BMD and markers of bone metabolism), not fractures. The strengths were the study design with all subjects starting the intervention at the beginning of the year; careful monitoring of the participants, being seen at regular intervals of 2 months; the precision of 25(OH)D measurements, standardized to NIST; and good subject retention throughout.

In conclusion, we found a small effect of vitamin D on BMD response over 1 year. There was a dose–response effect on PTH and serum calcium, albeit small, but not on any of the other measured markers including 1,25(OH)₂D and bone turnover markers.

### Disclosures

All authors state that they have no conflicts of interest.

### Acknowledgments

Trial registration: Vitamin D effects on cardiovascular disease risk (VICTOry) study at controlled-trials.com as ISRCTN20328039 (http://controlled-trials.com/ISRCTN20328039/). This work was funded partly by the UK Food Standards Agency, the Department of Health, and the National Osteoporosis Society.

We thank Prof Roger Francis (University of Newcastle, UK) for his role as Trial Steering Committee Chair; Prof Juliet Compston (University of Cambridge, UK) and Prof Bernard Keavney and Dr Mark Pearce (both of University of Newcastle, UK) as members of the Data Monitoring Committee; and the following staff at the University of Aberdeen, UK: Mrs Lismy Cheripelli, Registered General Nurse who assisted with the volunteer visits; Mrs Lana Gibson and Mrs Jennifer Scott for performing dual-energy X-ray absorptiometry scans; Ms Kelly Dean and Mrs Denise Tosh for bone marker measurements; Mrs Gladys McPherson (Health Services Research Unit), who provided the randomization service; and Ms Katrina Galbreith for help with data entry. Authors’ roles: HMM (principal investigator) had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. She was involved in the study design, study management, and the writing of the manuscript. ADW was responsible for the day-to-day running of the study and involved in the interpretation of the study data. LSA offered statistical advice and interpretation. AJB had responsibility for the volunteers’ welfare and gave assistance with the study design. WDF offered analysis and interpretation of measurements for 25-hydroxyvitamin D, PTH, and FGF23. AM contributed to the study design and interpretation of physical activity data. DMR (director of the Aberdeen Prospective Osteoporosis Screening Study from where the volunteers were recruited) contributed to the study design and interpretation. KS (the research nurse in charge of the study volunteers) contributed to the study design. WGS was responsible for routine biochemistry tests, including serum calcium and interpretation. FT was involved in study design and interpretation. HIM, ADW, AM, FT, and DMR were involved in obtaining study funding. All authors critically appraised the manuscript.

Role of the sponsor: The sponsor, Research and Innovation, University of Aberdeen, UK, was responsible for confirming proper arrangements to initiate, manage, monitor, and finance this RCT as designated by the Scottish Executive Health Department Research Governance Framework for Health and Community Care and the Department of Health Research Governance Framework for Health and Social Care 2nd Edition (2006)—Confirmation of the Role of Sponsor.
References


41. Sanders KM, Stuart AL, Williamson EJ, Simpson JA, Kotowicz MA, Young D, Nicholson GC. Annual high-dose oral vitamin D and falls and fractures in older women: a randomized controlled trial. JAMA. 2010;303(18):1815–22.
Effect of selenium supplementation on CD4+ T-cell recovery, viral suppression and morbidity of HIV-infected patients in Rwanda: a randomized controlled trial.
AIDS. 2015.
Effect of selenium supplementation on CD4⁺ T-cell recovery, viral suppression and morbidity of HIV-infected patients in Rwanda: a randomized controlled trial


Objective: To examine the effect of selenium supplementation on CD4⁺ T-cell counts, viral suppression, and time to antiretroviral therapy (ART) initiation in ART-naïve HIV-infected patients in Rwanda.

Methods: A multicenter, double-blinded, placebo-controlled, randomized clinical trial was conducted. Eligible patients were HIV-infected adults (≥21 years) who had a CD4⁺ cell count between 400 and 650 cells/μl (ART eligibility was ≤350 cells/μl throughout the trial), and were willing to practice barrier methods of birth control. Patients were randomized to receive once-daily 200 μg selenium tablets or identical placebo. They were followed for 24 months with assessments every 6 months. Declines in CD4⁺ cell counts were modeled using linear regressions with generalized estimating equations and effect modification, and the composite outcome (ART eligible or ART initiation) using Cox proportional-hazards regression, both conducted with intention to treat.

Results: Of the 300 participants, 149 received selenium, 202 (67%) were women, and median age was 33.5 years. The rate of CD4⁺ depletion was reduced by 43.8% [95% confidence interval (CI) 7.8–79.8% decrease] in the treatment arm – from mean 3.97 cells/μl per month to mean 2.23 cells/μl per month. We observed 96 composite outcome events – 45 (47%) in the treatment arm. We found no treatment effect for the composite outcome (hazard ratio 1.00, 95% CI 0.66–1.54) or viral suppression (odds ratio 1.18, 95% CI 0.71–1.94). The trial was underpowered for the composite outcome due to a lower-than-anticipated event rate. Adverse events were comparable throughout.

Conclusions: This randomized clinical trial demonstrated that 24-month selenium supplementation significantly reduces the rate of CD4⁺ cell count decline among ART-naïve patients.

Keywords: CD4⁺ cells, HIV/AIDS, randomized clinical trial, selenium

© 2015 Wolters Kluwer Health, Inc. All rights reserved.

AIDS 2015, 29:000–000

Copyright © 2015 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.
Introduction

HIV infection compromises the nutritional status of the infected individuals, and poor nutritional status can accelerate progression of the disease [1]. The relationship between immune function and nutritional supplementation has been well described [2–5]. Studies have reported high rates of nutrient deficiencies early in the course of HIV infection [6–8].

Among HIV-infected persons not receiving antiretroviral therapy (ART), observational studies have shown that low or deficient serum concentrations of several micronutrients, including the trace mineral selenium, are associated with low CD4⁺ T-cell counts, advanced HIV-related diseases, faster disease progression, or HIV-related mortality [9–23]. Selenium is incorporated into a number of biologically active selenoproteins and is an essential element of glutathione peroxidase (GPX), which plays an important role in endogenous antioxidant defense [24,25]. Selenium is also an essential factor in maintaining host immune competence, and it has been shown that optimal levels decrease the host’s susceptibility to viral pathogenesis [26–30]. Selenium deficiency, as indicated by low plasma selenium concentrations, is common in HIV-infected individuals [31,32], particularly in areas of the world with low selenium levels in the soil, as is true in many regions of sub-Saharan Africa [33–35].

Evidence of the effect of selenium supplementation from clinical trials is limited in both developed and developing countries. Three randomized clinical trials (RCTs) have been conducted to assess the individual association of selenium supplementation on HIV viral load and CD4⁺ T-cell count [36–38]. The first trial, conducted in Miami by Hurwitz et al. [37], found that selenium supplementation of 200 μg daily significantly suppressed the progression of HIV viral burden and indirectly improved CD4⁺ T-cell count after 9 months of treatment. The second trial, conducted in Tanzania by Kupka et al. [38], found that selenium supplementation of 200 μg daily provided to HIV-infected pregnant women before and after pregnancy (between 12 and 27 weeks of gestation and 6 months after birth) had no significant effect on HIV viral load or CD4⁺ T-cell count, but did significantly lower the risk of infant death. A third trial, a factorial design by Baum et al. [36] in Botswana, found no significant effect of selenium supplementation on the rate of depletion to 200 cells/μl among patients starting with a CD4⁺ T-cell count above 350 cells/μl. The authors did, however, find a significant event rate decrease when combined with multivitamins [36].

It is important to recognize the inherent differences in their design, setting, and populations of these trials when drawing inferences. Additional evidence from other settings and populations is required to more accurately determine the effect of selenium supplementation on HIV progression in HIV-infected individuals. Therefore, we conducted an RCT to examine the effect of selenium supplementation on CD4⁺ T-cell counts, viral suppression, and time to ART initiation in HIV-infected patients who are not yet on ART. We conducted our trial in Rwanda.

Methods

The present study is a 24-month, multicenter, patient and provider-blinded, randomized, placebo-controlled clinical trial, involving 300 pre-ART HIV-infected patients in Rwanda. We a priori calculated our sample size based by assuming a 20% reduction in CD4⁺ T-cell count depletion. This trial has been registered with ClinicalTrials.gov under the registration number NCT01327755.

Patients were recruited at three health facilities that offer care and treatment for HIV/AIDS patients in Rwanda. These facilities were chosen due to the feasibility of recruiting all patients within a short period and the feasibility of coordination. Patient eligibility was restricted to: HIV-infected adults (21 years of age and older at study enrollment), who were not yet ART-eligible based on Rwanda guidelines for ART initiation, had a CD4⁺ T-cell count between 400 and 650 cells/μl, were willing to practice barrier methods of birth control at all times, and be able to provide written informed consent. The CD4⁺ cell count at baseline was considerably above the Rwandan guidelines for initiation of ART (≥350 cells/μl as of 2012). Eligible patients were identified from pre-ART registers. Participants were enrolled and followed for 2 years. Study assessments occurred at baseline, 6, 12, 18, and 24 months.

Patients were excluded if they intended on transferring out of the clinic catchment area before the study ended and/or if they were scheduled to start ART. Patients with psychiatric health concerns and pregnant women were also excluded.

Randomization

The randomization flowchart is shown in Fig. 1. Participants were randomized using a simple randomized block design to receive either selenium or an identically appearing placebo to be taken once daily for 24 months. The research department of the treatment supplier prepared the randomization schedule. Study participants were identified by unique study identification numbers and were assigned a specific allocation number. An unblinded list was provided to the treatment provider and to the independent statistician for the drug safety monitoring board.

Intervention

The trial intervention consisted of once-daily tablets containing 200 μg of selenium in the form of
selenomethionine containing selenium yeast. The control arm received an identical placebo. To ensure optimal adherence, participants received adherence counseling at baseline and when picking up refills on a monthly basis. Additional adherence counseling was provided to patients who had sub-optimal adherence.

The Rwandan Ministry of Health recommends the use of cotrimoxazole – a sulfonamide antibiotic combination of trimethoprim and sulfamethoxazole used for the treatment of a variety of bacterial infections – for all HIV-infected patients. Therefore, all participants also received cotrimoxazole, irrespective of experimental assignment. Participants who did not return to the clinic as scheduled were followed up at home and received optimal adherence counseling.

Outcomes and study measures

The primary outcome measures for this study was change in CD4⁺ T-cell counts, and a composite of CD4⁺ T-cell depletion to 350 cells/µl (as confirmed by two consecutive measures), or start of ART, or the emergence of a documented CDC⁺-defined AIDS-defining illness. For analyses of the CD4⁺ T-cell count changes, patients were censored after ART initiation. Women who initiated ART through prevention of mother-to-child transmission programs prior to reaching other endpoints the composite outcome were censored at time of pregnancy because pregnancy is a mechanism by which ART is initiated independent of immunological failure.

Secondary outcomes included: viral suppression; mortality; and adverse events. This study used the standard level of expedited adverse event reporting as defined in the Division of AIDS (DAIDS) of the National Institute of Allergy and Infectious Diseases (NIAID) Manual for Expedited Reporting of Adverse Events (Version 2.0, January 2010). Adverse event follow-up was reported on a standardized form during the protocol-defined reporting period. After the end of the protocol-defined adverse event-reporting period, sites were asked to report serious, unexpected, clinical suspected adverse drug reactions or if the study site staff becomes aware of the event on a passive basis (e.g. from publicly available information).

Trained nurses used structured questionnaires to collect data on patient demographics at baseline. Additionally, at baseline and at each follow-up visit, a questionnaire was used to collect information on psychosocial factors, access to care and treatment, attitudes towards and experiences with the supplementation, quality of life, self-efficacy, nutrition, opportunistic infections, and adherence to the study protocol. The questionnaire was available in both English and Kinyarwanda. Nurses also collected clinical data at baseline and at each follow-up visit, including information on height, weight, and blood.

Analysis

Baseline demographic and clinical characteristics were tabulated and compared using Fisher’s exact test for
dichotomous outcomes and Wilcoxon’s rank-sum test for continuous outcomes. Statistical significance was assessed at the two-sided 5% level unless otherwise indicated. All analyses were based on the intention-to-treat (ITT) approach using the randomized treatment assignment. All available data were used and missing data were removed.

To examine the effect of selenium supplementation on CD4⁺ T-cell decay, we used linear regression to model change from baseline (i.e. all patients were assigned a change of 0 at baseline). The model included both time in study and treatment group, with an effect modification on time-by-treatment group. Generalized estimating equations (GEEs) were used to account for the repeated-measures nature of the data. Assumptions for linear regression, including homogeneity and normality of residuals, were assessed graphically and met.

Both time-to-event analyses and simpler contingency table analyses were used to determine whether selenium supplementation could delay the initiation of ART. Survival analysis was carried out by way of Cox proportional-hazards and Kaplan–Meier curves. Only two models were considered. The first was a simple model by which the only explanatory variable was treatment, and the second was an explanatory model with the addition of self-reported adherence to treatment (measured as all pills taken). As self-reported adherence was measured over time, we used it as a time-varying covariate. Moreover, we used baseline CD4⁺, age, sex, viral load, and other measures of adherence to generate multiple imputations to overcome the missingness in the adherence. These analyses were then repeated for unadjusted events (a single CD4 cell count event). All conditions for survival analysis were verified for homogeneity and normality of residuals, were assessed graphically and met.

For this secondary outcome of viral load, we considered observed suppression as a measured viral load less than 20 copies/μL. Missing viral loads were considered ‘not observed as suppressed’ (missing equals failure). Viral loads were measured at three time points: baseline, 12 months, and 24 months. Given that there were only two follow-up points, logistic regression was favored over survival analysis. As such, we used GEEs to account for repeated measurements on the same individual. Finally, we removed observation following ART initiation, as this would interfere with the effects of selenium supplementation on viral suppression. Two models were fit – one with only treatment as a predictor and the other with time of measurement.

All analyses were conducted in SAS 9.3 (Cary, North Carolina, USA) and in R version 3.0.2 (Vienna, Austria).

Ethics

The present trial received approval from the institutional review boards of the Canadian College of Naturopathic Medicine and Wilfred Laurier University in Canada, and the National Ethics Committee (NEC) in Rwanda.

Results

Between September 2010 and January 2011, 300 patients were identified as eligible and randomized in this study. The study was conducted between January 2011 and January 2013 (24 months follow-up). Figure 1 shows the randomization, loss to follow-up, and censoring due to pregnancy over the study period. Selenium supplements were provided to 151 participants. Over the 24-month study period, 18 patients were lost to follow-up – 9 within 6 months of study initiation, 4 more between 6 and 12 months into the study, 3 more in the next 6-month period, and finally 2 in the final 6 months. Reasons for loss to follow-up included accidental death, moving outside the study area, and unknown. The loss to follow-up counts did not include women who became pregnant and initiated ART through prevention of mother-to-child transmission programs. Pregnancy was nonetheless used as a censoring mechanism. Only two deaths were experienced, one in each arm, and neither appeared to be treatment-related.

Table 1 presents baseline characteristics of the study participants. There were 202 (67%) women, and the median age among participants was 33.5 years. The median baseline CD4⁺ cell count was 540 cells/μL with an interquartile range (IQR) from 468 to 627 cells/μL. The base 10 logarithm of viral load at baseline was 3.87 (IQR 3.15–4.45). In total, 84 (28%) patients reported not using barrier methods of birth control, which explains the 33 pregnancies observed over the 24-month study period. The duration of follow-up was 24 months for all but 19 patients.

Changes in CD4⁺ T-cell counts were heterogeneous, with a SD of 120.3 cells/μL. Table 2 summarizes the linear regression with GEEs used to model change in CD4⁺ according to the treatment group. The average rate of CD4⁺ decline among patients using placebo was 3.97 cells/μL per month [95% confidence interval (CI) 3.03, 4.91]. The rate of CD4⁺ decline was reduced by 43.8% (95% CI 7.8–79.8) among patients using selenium supplementation. Figure 2 presents the estimated regression lines for each treatment group and suggests a difference of approximately 40 cells in decline at the end of the study period in favor of the treatment group over the placebo group.

For the composite endpoint, a total of 86 events were observed after censoring for pregnancy (Table 3). None of the outcomes were an AIDS-defining illness. Most were CD4⁺ cell count depletion to below 350 cells/μL. Slightly more events occurred in the treatment group (51.2%), leading to a Fisher’s exact P value of 0.899.
When the definition of the composite outcome was relaxed to single measurement below 350 cells/μl, to account for missing second CD4⁺ measurements, a total of 115 composite outcomes were observed and 52 (45.2%) occurred in the selenium group. However, this comparison was also not statistically significant, with a Fisher’s exact \( P \) value of 0.192. Figure 3 shows the Kaplan–Meier estimates for reaching the composite endpoint. Table 4 shows the results of the Cox proportional-hazards regressions. In the analysis using the two consecutive low CD4⁺ measurements in the composite outcome, the hazard ratio comparing the treatment group to the placebo group was 1.00 (95% CI 0.66–1.53). The result was consistent when accounting for adherence. The sensitivity analysis using a relaxed definition for the CD4⁺ event led to similar results, but with a lower estimate hazard ratio (0.93) and a larger hazard ratio for being nonadherent.

For the secondary outcome of viral suppression, the effect of treatment in the unadjusted model was an odds ratio (OR) of 1.18 (95% CI 0.71–1.93) in favor of selenium. During the trial, the only serious adverse event (SAE) reported was a myocardial infarction, resulting in death in the treatment group. There was no evidence to suggest that this SAE was directly related to the treatment. With respect to the self-reported adverse events collected at every 6 months, there was minimal evidence of statistical differences between the treatment groups. The comparisons across treatment groups are presented in Table A1 (Supplemental Digital Content 1, http://links.lww.com/QAD/A679) of the ‘Web Appendix’. Patients taking the placebo were more likely to be anxious \( (P = 0.035) \). Patients taking selenium supplementation were more likely to report that sleep symptoms bothered them a lot, but less likely to report that it bothered them a little \( (P = 0.011) \). Both groups reported having no

### Table 1. Baseline demographics and risk factors.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Values</th>
<th>Total</th>
<th>Active count (%) or median (IQR)</th>
<th>Placebo count (%) or median (IQR)</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male</td>
<td>98</td>
<td>44 (29.1%)</td>
<td>54 (36.2%)</td>
<td>0.151</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>202</td>
<td>107 (70.9%)</td>
<td>95 (63.8%)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td>300</td>
<td>33.0 (28.0–39.0)</td>
<td>35.0 (28.0–41.0)</td>
<td>0.418</td>
</tr>
<tr>
<td>Marital status</td>
<td>Married or living with partner</td>
<td>180</td>
<td>90 (60.8%)</td>
<td>90 (60.4%)</td>
<td>0.916</td>
</tr>
<tr>
<td></td>
<td>Single</td>
<td>26</td>
<td>15 (10.1%)</td>
<td>11 (7.4%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Widowed</td>
<td>32</td>
<td>15 (10.1%)</td>
<td>17 (11.4%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Separated</td>
<td>41</td>
<td>19 (12.8%)</td>
<td>22 (14.8%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Divorced</td>
<td>18</td>
<td>9 (6.1%)</td>
<td>9 (6%)</td>
<td></td>
</tr>
<tr>
<td>Employment</td>
<td>No</td>
<td>132</td>
<td>65 (43.3%)</td>
<td>67 (45.3%)</td>
<td>0.585</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>165</td>
<td>84 (56%)</td>
<td>81 (54.7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Refused</td>
<td>1</td>
<td>1 (0.7%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>BMI at baseline</td>
<td></td>
<td>266</td>
<td>21.5 (19.8–23.7)</td>
<td>21.6 (20.0–24.3)</td>
<td>0.379</td>
</tr>
<tr>
<td>CD4⁺ at baseline</td>
<td></td>
<td>300</td>
<td>552 (470–636)</td>
<td>527 (465–610)</td>
<td>0.126</td>
</tr>
<tr>
<td>Viral load (log of)</td>
<td></td>
<td>268</td>
<td>3.8 (3.0–4.5)</td>
<td>3.9 (3.3–4.4)</td>
<td>0.324</td>
</tr>
<tr>
<td>Has had sex in past month</td>
<td>No</td>
<td>110</td>
<td>50 (33.3%)</td>
<td>60 (40.3%)</td>
<td>0.214</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>189</td>
<td>100 (66.7%)</td>
<td>89 (59.7%)</td>
<td></td>
</tr>
<tr>
<td>Number of partners in past 30 days</td>
<td>NA (skipped)</td>
<td>110</td>
<td>50 (33.3%)</td>
<td>60 (40.5%)</td>
<td>0.390</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>185</td>
<td>98 (65.3%)</td>
<td>87 (58.8%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3</td>
<td>2 (1.3%)</td>
<td>1 (0.7%)</td>
<td></td>
</tr>
<tr>
<td>Condom use in past 30 days</td>
<td>NA (skipped)</td>
<td>110</td>
<td>50 (33.3%)</td>
<td>60 (40.5%)</td>
<td>0.233</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>104</td>
<td>59 (39.3%)</td>
<td>45 (30.4%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sometimes</td>
<td>30</td>
<td>12 (8%)</td>
<td>18 (12.2%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Never</td>
<td>54</td>
<td>29 (19.3%)</td>
<td>25 (16.9%)</td>
<td></td>
</tr>
</tbody>
</table>

IQR, interquartile range; NA, not available.

### Table 2. Linear regression with generalized estimating equations.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Average CD4⁺ change (95% confidence interval)</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active vs. placebo</td>
<td>(-4.37 (-13.78, 5.04))</td>
<td>0.363</td>
</tr>
<tr>
<td>Time (per month)</td>
<td>(-3.97 (-4.91, -3.03))</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time adjustment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active vs. placebo</td>
<td>(1.74 \ (0.31, 3.17))</td>
<td>0.017</td>
</tr>
</tbody>
</table>

Fig. 2. Rate of CD4⁺ T-cell count decline across treatment groups.
symptoms, with approximately equal probability. When adjusting for multiple testing, none of these results were statistically significant at the 0.05 significance level. Overall, selenium and placebo supplements were very well tolerated, helping explain the high percentage of study completers.

Discussion

The present RCT on selenium supplementation in pre-ART HIV-positive patients in Rwanda provides evidence of the benefits of selenium supplementation with respect to reduced rates of CD4\(^{+}\) declines. Our study found a significant decrease in CD4\(^{+}\) depletion rates, but not on the combined composite events, including reaching a CD4\(^{+}\) point of less than 350 cells/\(\mu\)l, AIDS-defining illness, or death.

Our study has strengths and limitations. The strengths include our recruitment of patients with higher CD4\(^{+}\) levels than previous trials that provide evidence for supplementation at an earlier stage and prior to the use of antiretrovirals. Moreover, we monitored adherence and high retention rate as a result of a strong commitment to the study on the part of the site nurses. Limitations include that the event rate for the composite outcome was much lower than the 60% used for our power calculations. This resulted in an underpowered analysis for this outcome. Moreover, only 8 women were censored due to pregnancy in the placebo group compared to 15 in the treatment group. This unbalanced censoring may have biased the results in favor of the placebo treatment. This was the only observed factor by which the treatment groups differed following randomization. The different rates of censoring are not believed to have affected the CD4\(^{+}\) and viral load analyses.

Although the Miami trial provided strong evidence for suppression of viral burden and an indirect improvement of CD4\(^{+}\), Ross et al. [39] pointed out that these sub-analyses did not retain the original randomized treatment allocation. As such, these findings may be subject to bias not identified by Hurwitz et al. Particularly, analyses were performed in which participants in the selenium group who showed a large increase in serum selenium concentration (‘selenium responders’: defined as a change in serum selenium concentration between baseline and 9 months of above 3 SDs more than the mean change in the placebo group) were compared with ‘selenium nonresponders’ and with those allocated to placebo. Our findings were most closely observed in the Botswana trial, where selenium supplementation was shown to significantly reduce CD4\(^{+}\) decline, but with no impact on viral burden.

The outcome of this study provides further evidence of the benefit of low-cost micronutrient supplementation to HIV-positive patients. This is particularly important for patients living in resource-limited settings such as much of Africa. Although assistance has been received from international funding agencies in order to make the antiretroviral drugs available for widespread distribution and treatment of HIV/AIDS patients, it is still a major financial burden to these countries. This financial burden is compounded by escalating healthcare costs associated with a rise in noncommunicable diseases [33,40]. In a period of worldwide financial uncertainty, any low-cost treatment that will slow the progression of the disease

### Table 3. Contingency tables for composite outcome.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total</th>
<th>No</th>
<th>Yes</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Composite outcome</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active</td>
<td>151</td>
<td>107</td>
<td>44</td>
<td>0.899</td>
</tr>
<tr>
<td>Placebo</td>
<td>149</td>
<td>107</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Total (n)</td>
<td>300</td>
<td>214</td>
<td>86</td>
<td></td>
</tr>
</tbody>
</table>

Use only a single CD4\(^{+}\) measurement below 350 as event

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total</th>
<th>No</th>
<th>Yes</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active</td>
<td>151</td>
<td>99</td>
<td>52</td>
<td>0.192</td>
</tr>
<tr>
<td>Placebo</td>
<td>149</td>
<td>86</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>Total (n)</td>
<td>300</td>
<td>185</td>
<td>115</td>
<td></td>
</tr>
</tbody>
</table>

### Table 4. Survival analysis for the composite outcome.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Protocol data hazard ratio (95% CI)</th>
<th>Unadjusted data hazard ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple model</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active vs. placebo</td>
<td>1.00 (0.66–1.53)</td>
<td>0.93 (0.66–1.31)</td>
</tr>
<tr>
<td>Adjusted model</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active vs. placebo</td>
<td>1.00 (0.66–1.54)</td>
<td>0.94 (0.66–1.33)</td>
</tr>
<tr>
<td>Less-adherent vs. adherent</td>
<td>1.17 (0.70–1.96)</td>
<td>1.31 (0.83–2.02)</td>
</tr>
</tbody>
</table>

CI, confidence interval.

*aThis represents a sensitivity analysis by which a CD4\(^{+}\) event only required a single CD4\(^{+}\) below 350 cells/\(\mu\)l.
prior to the requirement for pharmacological intervention should be welcomed.

A growing concern in the treatment of HIV/AIDS in sub-Saharan Africa is the increase in drug resistance among those being treated for HIV/AIDS, particularly in regions where there had been an early roll out of ART [41]. A delay in progression of the disease brought about by low-cost micronutrient supplementation would be advantageous in modifying the rate of drug resistance at least at the level of the individual.

The HIV disease burden in sub-Saharan Africa is still important, in terms of incidence, social impact, and healthcare costs. On the basis of the outcome of this study, micronutrient supplementation with the trace mineral selenium may be something to consider in the overall treatment plan for HIV-positive patients in the pre-ART phase of care.

Acknowledgements

The present trial was sponsored by Global Benefit Canada. We also thank CanAlt Labs and Seroyal – a nutraceutical company – for supplying the supplement.

Rwanda Selenium Trial Authorship Group. Additional authors: Richard Smyth – Rwanda Selenium Supplementation Clinical Trial Team, Kigali, Rwanda; Heather Fay – Rwanda Selenium Supplementation Clinical Trial Team, Kigali, Rwanda; Donatille Habarurema – Rwanda Selenium Supplementation Clinical Trial Team, Kigali, Rwanda; Veneranda Mukarukundo – Rwanda Selenium Supplementation Clinical Trial Team, Kigali, Rwanda; Alice Umurerwa – Rwanda Selenium Supplementation Clinical Trial Team, Kigali, Rwanda; Cara Silva – Rwanda Selenium Supplementation Clinical Trial Team, Kigali, Rwanda; Dugdal Seely – Canadian College of Nutraceutical Medicine, North York, Ontario, Canada; Douglas J. McCready – Department of Economics, School of Business and Economics, Wilfrid Laurier University, Waterloo, Ontario, Canada; Steve Kanters – School of Population and Public Health, University of British Columbia, Vancouver, British Columbia, Canada; Edward J. Mills – Stanford Prevention Research Center, Stanford School of Medicine, Stanford, California, USA; Don Warren – Rwanda Selenium Supplementation Clinical Trial Team, Kigali, Rwanda.

Conflicts of interest
None declared.

References


Effect of selenium supplementation on CD4 T-cell recovery, viral suppression, morbidity and quality of life of HIV-infected patients in Rwanda: study protocol for a randomized controlled trial. Trials. 2011.
Effect of selenium supplementation on CD4 T-cell recovery, viral suppression, morbidity and quality of life of HIV-infected patients in Rwanda: study protocol for a randomized controlled trial

Julius Kamwesiga1, Vincent Mutabazi1, Josephine Kayumba1, Jean-Claude K Tayari1, Richard Smyth1, Heather Fay1, Alice Umurewa1, Marcel Baziruwiha1, Christian Ntizimira1, Antoinette Murebwayire1, Jean Pierre Haguma1, Julienne Nyiransabimana1, Donatille Habubreema1, Veneranda Mukurukundo1, Jean Bosco Nzabandora1, Pascal Nzamwita1, Ernestine Mukazayire1, Edward J Mills2*, Dugald Seely3, Douglas J McCready4 and Don Warren1

Abstract

Background: Low levels of serum selenium are associated with increased risk of mortality among HIV+ patients in East Africa. We aim to assess the effect of selenium supplementation on CD4 cell count, HIV viral load, opportunistic infections, and quality of life in HIV-infected patients in Rwanda.

Methods and Design: A 24-month, multi-centre, patient and provider-blinded, randomized, placebo-controlled clinical trial involving 300 pre-antiretroviral therapy (ART) HIV-infected patients will be carried out at two sites in Rwanda. Patients ≥ 21 years of age with documented HIV infection, CD4 cell count of 400-650 cells/mm³, and not yet on ART will be recruited. Patients will be randomized at each study site using a randomized block design to receive either the selenium micronutrient supplement or an identically appearing placebo taken once daily. The primary outcome is a composite of time from baseline to reduction of CD4 T lymphocyte count below 350 cells/mm³ (confirmed by two measures at least one week apart), or start of ART, or the emergence of a documented CDC-defined AIDS-defining illness. An intention-to-treat analysis will be conducted using stepwise regression and structural equation modeling.

Discussion: Micronutrient interventions that aim to improve CD4 cell count, decrease opportunistic infections, decrease HIV viral load, and ultimately delay initiation of more costly ART may be beneficial, particularly in resource-constrained settings, such as sub-Saharan Africa. Additional trials are needed to determine if micro-supplementation can delay the need for more costly ART among HIV-infected patients. If shown to be effective, selenium supplementation may be of public health importance to HIV-infected populations, particularly in sub-Saharan Africa and other resource-constrained settings.

Trial Registration: NCT01327755

Background

In sub-Saharan Africa, more than 30 million people are living with HIV/AIDS, malnutrition and food insecurity are endemic [1] HIV infection compromises the nutritional status of infected individuals and poor nutritional status can enhance progression of the disease [2]. The relationship between immune function and nutritional supplementation has been well described [3-6]. Studies have reported a high prevalence of nutrient deficiencies early in the course of HIV infection [7-9].

It is well understood that micronutrient deficiencies and HIV disease progression aggravate each other [10,11]. Among HIV-infected persons not receiving anti-retroviral therapy (ART), observational studies have shown that low or deficient serum concentrations of several micronutrients are associated with low CD4 cell
count, advanced HIV related diseases, faster disease progression, or HIV-related mortality [12-26]. Selenium is one essential nutrient necessary for endogenous antioxidant defense. Selenium deficiency, as indicated by low plasma selenium concentrations, is common among HIV-infected individuals [27,28].

While many observational studies on selenium in HIV-infected patients have been conducted in developed countries over the past decade, few have been conducted in sub-Saharan Africa. Clinical trial data on selenium supplementation is also limited in both developed and developing countries. There are two recent high-impact randomized clinical trials (RCTs) that have been conducted to assess the individual association of selenium supplementation on HIV viral load and CD4 cell count. The first trial, conducted in Miami by Hurwitz et al [29], found that selenium supplementation of 200 μg daily significantly suppressed the progression of HIV viral load and improved CD4 cell count after 9 months of treatment. The second trial, conducted in Tanzania by Kupka et al [30], found that selenium supplementation of 200 μg daily provided to HIV-infected pregnant women before and after pregnancy (between 12 and 27 weeks of gestation and 6 months after birth) had no significant effect on HIV viral load or CD4 cell count, but did significantly lower risk of infant death.

Although the results of these two clinical trials appear to contradict, it is important to recognize the inherent differences in their design, setting, and populations under study. Additional evidence from other settings and populations is still required to more accurately determine the effect of selenium supplementation on HIV viral load and CD4 cell count in HIV-infected individuals. Therefore, we have designed a randomized trial to examine the effect of selenium supplementation on CD4 cell counts in HIV-infected patients who are not yet on ART. This trial will take place in Rwanda, where an estimated 250,000 adults and children are living with HIV [31], of which only 50,000 are receiving ART [32].

Methods and Design
Funding
Global Benefit has sponsored this trial. The trial sponsors and investigators will make financial assurance statements to the concerned bodies (Ministry of Health’s National Ethics Commission, National Research Commission) on the availability of funds for the completion of the trial.

Registration
This trial has been registered with ClinicalTrials.gov. The registration number is NCT01327755.

Study Design
This study is a 24-month, multi-centre, patient, provider and analyst-blinded, randomized, placebo-controlled clinical trial involving 300 pre-ART HIV-infected patients in Rwanda. (See Figure 1 for a study flow diagram.)

Objectives and Hypothesis
The primary outcome of the study is time from baseline to reduction of CD4 T lymphocyte count below 350 cells/mm³ (confirmed by two measures at least one week apart), or start of ART, or the emergence of a documented CDC-defined AIDS-defining illness. We hypothesize that selenium supplementation in pre-ART patients will improve CD4 cell counts, decrease opportunistic infections, decrease HIV viral load, and delay ART initiation.

Setting and Participants
Patients will be recruited at two purposely-selected health facilities that offer care and treatment for HIV/AIDS patients in Rwanda. These facilities have been chosen due to the feasibility of recruiting all patients within a 3-4 month period and the feasibility of coordination.

Patient eligibility will be restricted to HIV-infected adults 21 years of age and older at study enrolment. Only patients not yet eligible for ART will be included. Only patients with CD4 cell count between 400 and 650 cells/mm³ will be selected because they are at similar immunological level and hence they will not be eligible to start ART treatment at the start of the study. Eligible participants must also be willing to practice a barrier method of birth control at all times. Written informed consent will be required from patients to participate in this study.

Eligible patients will be identified from patient registers. These patients will be informed about the study during a regular scheduled clinic visit or through home visits by site staff not otherwise involved in data collection. Patients who are interested in the study will be provided with further details and consent procedures. Those fitting the inclusion criteria will be enrolled and followed for 2 years. Study assessments will occur at baseline, 6, 12, 18, and 24 months.

Patients will be excluded if they are intending to be transferred out of the clinic catchment area before study ends and/or if they are scheduled to start ART. Moribund patients, pregnant women, and those unable or not wanting to commit to barrier method of birth control will also be excluded.

Randomization
Participants will be randomized using a simple randomized block design to receive either selenium or an identically appearing placebo to be taken once daily for 24 months. The Rwandan Ministry of Health recommends
the use of co-trimoxazole, a sulfonamide antibiotic combination of trimethoprium and sulfamethoxazole used for the treatment of a variety of bacterial infections, for all HIV-infected patients. Therefore, all participants will also receive co-trimoxazole irrespective of experimental assignment. Participants who do not return to the clinic as scheduled will be followed up at home.

Study participants will be identified by a unique study identification number. The randomization schedule will be prepared by the product manufacturer, Seroyal, and the randomization sequence will be concealed from the study investigators and providers. Study participants will be assigned a specific allocation number. An unblinding list will be kept at the site of the manufacturer and will be provided as needed to the Data and Safety Monitoring Officer (DS). Site study personnel (investigator and clinical personnel monitoring the safety and laboratory assay results) and study participants will be blinded with respect to the allocation. Unblinding of an individual study participant will be indicated in the event of a medical emergency where the clinical management/medical treatment of the study participant could be altered by knowledge of the group assignment allocation of the investigational product.

### Intervention

The trial intervention will consist of capsules containing 200 mcg of selenium in the form of selenium yeast, which contains selenomethionine. Capsules will be

---

**Figure 1 Study flow diagram** This figure displays the intended recruitment and measurement points in this trial.
provided in bottles of 30 (one months supply) and participants will be instructed to take one capsule daily. Selenium will be stored in a dry cool location during the course of the trial. Shelf life of the product is well beyond the two-year time period expected for the trial duration. To ensure optimal adherence, participants will receive adherence counseling at baseline and when picking up refills on a monthly basis. Additional adherence counseling will be provided to patients who have sub-optimal adherence.

Selenium capsules are supplied by Seroyal, a Canadian Nutraceutical company. The sponsors of the trial and the investigators will be responsible for ensuring that the investigational pharmaceutical product is safe. Comparator products (placebo) supplied for the clinical trial will be of proven quality and will be available to be verified by the National Bureau of Standards. Records will be kept of information about the shipment, delivery, receipt, storage, return, and destruction of any remaining pharmaceutical products. The investigators will not supply the investigational product to any person not targeted to receive it.

Outcomes
The primary outcome of the study is a composite outcome involving reduction of CD4 T lymphocyte count to below 350 cells/mm$^3$ (confirmed by two measures at least one week apart), or start of ART, or the emergence of a documented CDC-defined AIDS-defining illness. Secondary outcomes include viral suppression at 6, 12, 18, and 24 months; quality of life; weight gain; presence of opportunistic infections and mortality.

Measurement of Outcomes and Other Variables of Interest

Patient Interviews

Trained nurses will use a structured questionnaire to collect data on patients’ demographics at baseline. Additionally, at baseline and at each follow-up visit, a questionnaire will be used to collect information on psychosocial factors, access to care and treatment, attitudes towards and experiences with the supplementation, quality of life, self-efficacy, nutrition, opportunistic infections, and adherence to the study protocol. The questionnaire will be available in English and Kinyarwanda. Nurses will also use a data abstraction tool at baseline and at each follow-up visit to obtain information on height, weight, blood pressure and other clinically relevant information.

HIV Viral Load and CD4 Cell Count

At baseline, and at month 6, 12, 18 and 24, blood draws will be done for assessment of CD4 cell count and HIV viral load. Blood samples will be collected into EDTA tubes (Becton Dickinson, San Jose, CA, USA). The viral load samples will be transported and analyzed at the National Reference Laboratory within 4 hours; plasma will be separated from cells by centrifugation at 200 g, aliquoted and stored at -70°C until the real time PCR will be performed using Cobas TaqMan 48 (Roche Diagnostic Systems, NJ, USA) that has a detection limit of 40 RNA copies/ml. The RNA will be extracted from the plasma with chloroform, followed by alcohol precipitation and dilution with the HIV Monitor specimen diluent (100 μl). Amplification and detection of the extracted RNA (40 μl) will be performed in accordance with the manufacturer’s instructions. CD4 cell count will be measured at the National Reference Lab, or the site laboratory, using FACS Count (Becton Dickinson Immunocytometry).

Sample Size Calculation

We chose our CD4 depletion event rate based on the work of the CASCADE cohort which displays an average CD4 depletion of 114 (32-229) cells/μl per year and where 54% of individuals had a decline > 100 cells/μl per year if CD4 evaluations were rare, as in Rwanda [33]. We expect that the majority of participants in our study, about 60%, will enter the study with a CD4 at risk of reaching 350 cells/μl within the first year as most evidence demonstrates that patients in East Africa initiate treatment due to symptomatic HIV with a median CD4 of 141 to 169 (depending on source). Thus, patients with a high CD4 will be somewhat more rare to enroll [34,35].

We applied several sample sizes to display that even small changes in the relative risk of the intervention will yield a large impact on the required sample size (see Table 1). Our three sample size assumptions are based on the likelihood of an intervention delivering a small, moderate or large effect (relative risk reduction of 10, 25, and 50%). Given that we do not have strong evidence from previously completed trials, we are assuming the event of depleting CD4 status will occur in 60% of control patients at one year and 60% of experimental patients at one year if the intervention delivers no effect. Table 1 provides the estimates required for power of 80% and an alpha of 0.05.

We employed several methods to make these calculations. The below estimates are based on Markov Chain Monte Carlo Methods whereby we explored the impact

<table>
<thead>
<tr>
<th>Table 1 Sample Size Calculation estimates</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRR</td>
</tr>
<tr>
<td>-----</td>
</tr>
<tr>
<td>10%</td>
</tr>
<tr>
<td>25%</td>
</tr>
<tr>
<td>50%</td>
</tr>
</tbody>
</table>
of adherence/retention to the intervention and examined the differing possible risks of an underlying disease event. We performed 5,000 simulations for each estimate. As we employ informed estimates, using a Bayesian profile, our estimates may differ slightly compared with other software approaches. However, they should not differ to an important amount.

Based on these calculations and our resources, we aim to enroll 300 patients in the trial. This leads to an RRR slightly lower than 25% and represents a very important reduction in events. Recognizing that no single trial can provide definitive evidence of effectiveness, this trial will contribute to the overall evidence of selenium supplementation for CD4 maintenance [36,37]. If control patients demonstrate lower rates of compliance to the intervention due to a lack of effects of, say, 20%, the power is increased to 96% and an alpha of 0.25. At the conclusion of our trial, we will conduct a meta-analysis to determine the overall power that current evidence contributes to answering this clinical question.

Analysis Plan

Analysis will be conducted jointly by the study team in Rwanda and in Canada using standard statistical software. The analysis and reporting of the results will follow the CONSORT guidelines [38]. The statistician/data analyst will be blinded to the study group. The process of patient selection and flow throughout the study will be summarized using a flow-diagram (See Figure 1). The analysis results of patient demographics and baseline outcome variables (both primary and secondary) will be summarized using descriptive summary measures: expressed as mean (standard deviation) or median (minimum-maximum) for continuous variables and number (percent) for categorical variables. We will adopt an intention-to-treat principle to analyze all outcomes, meaning that data from participants will be analyzed according to the group to which they were randomized even if they do not receive the allocated intervention. We will also use multiple-imputation to handle missing data. We will use the T-test for comparing groups on continuous outcomes and the chi-squared test for binary outcomes. We will consider a threshold of 0.05 as statistical significance. For all group comparisons, the results will be expressed as effect (risk ratio for binary outcomes), corresponding two-sided 95% confidence intervals and associated p-values. P-values will be reported to three decimal places with values less than 0.001 reported as < 0.001. Because the primary outcome is a composite outcome, we will assess heterogeneity between the included outcomes [39,40]. Further, adjusted analyses using the following baseline covariates (age, gender, nutritional status) will be performed using regression techniques to investigate the residual impact of key baseline characteristics on the outcomes. Goodness-of-fit will be assessed by examining the residuals for model assumptions and chi-squared test of goodness-of-fit. All analyses will be performed by a professional statistician.

Adverse Events

This study will use the standard level of expedited adverse event (AE) reporting as defined in the Division of AIDS (DAIDS) AE Manual. At this level, this study will report all AEs following any exposure to the investigational product. AE follow-up will be reported on a standardized form during the protocol-defined AE reporting period, which will be the entire study duration for an individual participant (from study enrollment until study completion or study discontinuation of a participant for any reason). After the end of the protocol-defined AE reporting period, sites will report serious, unexpected, clinical suspected adverse drug reactions or if the study site staff becomes aware of the event on a passive basis (e.g. from publicly available information).

AEs will be managed in accordance with good medical practices by the clinical study team who will assess and treat the study participant as appropriate, including referral. All study participants experiencing AEs, regardless of severity, will be followed until satisfactory resolution, return to baseline, or until the toxicity is presumed to be irreversible. If at the end of the study, an AE (including clinically significant lab abnormality) which is considered possibly, probably or definitely related to the investigational product is unresolved, follow-up will continue until resolution if possible and the study participant will be referred. If treatment and medical care is required as a result of harm caused by the investigational product or study procedures, this will be provided free of charge to the participant.

Ethical Considerations

This trial will be conducted in compliance with the protocol approved by the institutional review boards of the Canadian College of Naturopathic Medicine and Wilfred Laurier University in Canada, and the National Ethics Committee (NEC) in Rwanda. No deviation from the protocol will be implemented without the prior review and approval of the IRB except where it may be necessary to eliminate an immediate hazard to a participant. In such case, the deviation will be reported to the IRB as soon as possible.

A signed consent form will be obtained from each participant. The consent form describes the purpose of the trial, the procedures to be followed and the risks and benefits of participation. A copy of the consent form will be offered to the participant. Trial participants will receive 1,000 Rwandan Francs (equivalent to ~ $1.67 US dollars) per month for their travel to and from the clinic for interviews, lab work-up and picking up their supplement. In addition to this compensation, in the event that the study shows benefit, Global Benefit
(the trial funder) is committed to supplying free supplementation to all participants in the trial for a period of at least one year following study completion.

Quality Control and Quality Assurance

The sponsor and investigators will be responsible for implementing and maintaining quality assurance and quality control systems with written standard operating procedures to ensure that the trial is conducted and data are generated, documented, and reported in compliance with the protocol, good clinical practice, and the applicable regulatory requirements. The sponsor will also be responsible for securing agreement from all involved parties to ensure direct access to all trial related sites, source data and documents, and reports for the purpose of monitoring and auditing by the sponsor, and inspection by domestic and foreign regulatory authorities.

Discussion

Micronutrient interventions that aim to improve CD4 cell count, decrease opportunistic infections, decrease HIV viral load, and ultimately delay initiation of more costly ART may be beneficial, particularly in resource-constrained settings, such as sub-Saharan Africa. It has been shown that selenium is deficient in HIV-infected populations [27,28]. For instance, a study conducted among HIV-infected children in Rwanda found that close to 40% had sub-optimal levels of selenium [41].

Data assessing the efficacy of selenium supplementation in randomized controlled trials is limited. Additional trials are needed to determine if selenium supplementation can delay the need for ART among HIV-infected patients. If shown to be effective, selenium supplementation may be of great public health importance to HIV-infected populations, particularly in sub-Saharan Africa and other resource-constrained settings.

List of Abbreviations


Acknowledgements

We thank Seroyal for creating and donating the selenium and placebo tablets. Funding: We thank Global Benefit Canada for funding.

Author details

1 Rwanda Selenium Supplementation Clinical Trial Team, Kigali, Rwanda. 2 Interdisciplinary School of Health Sciences, Faculty of Health Sciences, University of Ottawa, Ottawa, Ontario, Canada. 3 Canadian College of Naturopathic Medicine, North York, Ontario, Canada. 4 Department of Economics, School of Business and Economics, Wilfrid Laurier University, Waterloo, Ontario, Canada.

Authors’ contributions

JK, VM, JK, JCK, RS, HF, AU, MB, CN, AM, JPH, JN, DH, VM, JBN, PN, EM, EJM, DS, DJM, DW conceived and designed the study. JK, VM, JK, JCK, RS, HF, AU, MB, CN, AM, JPH, JN, DH, VM, JBN, PN, EM, EJM, DS, DJM, DW interpreted the results of the literature search. JK, VM, JK, JCK, RS, HF, AU, MB, CN, AM, JPH, JN, DH, VM, JBN, PN, EM, EJM, DS, DJM, DW drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 10 June 2011 Accepted: 13 August 2011 Published: 13 August 2011

References


doi:10.1186/1745-6215-12-192

Cite this article as: Kamwesiga et al. Effect of selenium supplementation on CD4 T-cell recovery, viral suppression, morbidity and quality of life of HIV-infected patients in Rwanda: study protocol for a randomized controlled trial. Trials 2011 12:192.
A placebo-controlled trial of acetyl-l-carnitine and α-lipoic acid in the treatment of bipolar depression.
A placebo-controlled trial of acetyl-l-carnitine and α-lipoic acid in the treatment of bipolar depression.
A Placebo-Controlled Trial of Acetyl-\(\text{l}\)-Carnitine and \(\alpha\)-Lipoic Acid in the Treatment of Bipolar Depression

Brian P. Brennan, MD, MMSc,*†‡ John Eric Jensen, PhD,†§ James I. Hudson, MD, ScD,*† Caitlin E. Coit, BA,* Ashley Beaulieu, BA,* Harrison G. Pope, Jr, MD, MPH,*† Perry F. Renshaw, MD, PhD,** and Bruce M. Cohen, MD, PhD†‡

**Background:** Bipolar disorder may be associated with mitochondrial dysfunction. Therefore, agents that enhance mitochondrial functioning may be efficacious in bipolar disorder. We performed a randomized placebo-controlled trial of the mitochondrial enhancers acetyl-\(\text{l}\)-carnitine (ALCAR) and \(\alpha\)-lipoic acid (ALA) in patients with bipolar depression, and assessed markers of cerebral energy metabolism using phosphorous magnetic resonance spectroscopy.

**Methods:** We administered ALCAR (1000–3000 mg daily) plus ALA (600–1800 mg daily) or placebo for 12 weeks to 40 patients with bipolar depression and obtained imaging data at baseline, week 1, and week 12 of treatment in 20 patients using phosphorous 3-dimensional chemical-shift imaging at 4 T. Statistical analysis used random effects mixed models.

**Results:** We found no significant difference between ALCAR/ALA and placebo on change from baseline in the Montgomery-Asberg Depression Rating Scale in both the longitudinal (mean difference [95% confidence interval], \(-1.4 [-6.2 to 3.4], P = 0.58\)) and last-observation-carried-forward (\(-3.2 [-7.2 to 0.9], P = 0.12\)) analyses. ALCAR/ALA treatment significantly reduced phosphocreatine levels in the parieto-occipital cortex at week 12 (\(P = 0.002\)). Reduction in whole brain total nucleoside triphosphate levels from baseline to week 1 was associated with reduction in Montgomery-Asberg Depression Rating Scale scores (\(P = 0.02\)) in patients treated with ALCAR/ALA. However, this was likely a chance finding attributable to multiple statistical comparisons.

**Conclusions:** Treatment with ALCAR and ALA at the dose and duration used in this study does not have antidepressant effects in depressed bipolar patients and does not significantly enhance mitochondrial functioning in this patient group.

**Key Words:** bipolar disorder; depression; mitochondria; acetyl-\(\text{l}\)-carnitine, \(\alpha\)-lipoic acid

(J Clin Psychopharmacol 2013;33: 00–00)

---

**Biological Psychiatry Laboratory, McLean Hospital, Belmont; †Department of Psychiatry, Harvard Medical School, Boston; ‡Shervert Frazier Research Institute, and †Brain Imaging Center, McLean Hospital, Belmont, MA; and ||Department of Psychiatry, University of Utah School of Medicine, Salt Lake City, UT.**

Received April 18, 2012; accepted after revision January 2, 2013.

Reprints: Brian P. Brennan, MD, MMSc, McLean Hospital, 115 Mill St, Belmont, MA 02478 (e-mail: bbrennan@partners.org).

This study was supported by grants from the Stanley Medical Research Institute, the Sidney R. Baer, Jr. Foundation through a NARSAD Young Investigator Award (B.P.B.), and the National Institutes on Drug Abuse (NIDA) Grant T32-DA07252 (B.P.B.). In addition, B.P.B. was supported by the Clinical Investigator Training Program (CITP) through the Beth Israel Deaconess Medical Center-Harvard/MIT Health Sciences and Technology, in collaboration with Pfizer Inc and Merck & Co.

Copyright © 2013 by Lippincott Williams & Wilkins

ISSN: 0271-0749

DOI: 10.1097/JCP.0b013e31829a8375

---

Bipolar disorder is a common and often disabling mental illness. The depressive phase of bipolar disorder frequently dominates the illness and results in significant morbidity and mortality. Several pharmacologic treatments including lithium, anticonvulsants, and antipsychotic medications have demonstrated efficacy in the depressed phase of bipolar disorder, but many patients fail to respond or cannot tolerate first-line mood-stabilizer treatments. Furthermore, traditional monoaminergic antidepressant agents may not outperform mood stabilizers alone in such patients. Thus, novel treatment strategies for bipolar depression are needed.

Recent research suggests that abnormal mitochondrial functioning may contribute to bipolar disorder. Although this dysfunction is insufficient to produce a systemic metabolic disorder, it could produce a brain disorder, because the brain requires much larger amounts of energy than other organs. Evidence for this hypothesis comes from studies demonstrating a variety of findings in patients with bipolar disorder including (1) abnormalities in several neurochemical markers of cerebral energy metabolism on both proton (\(\text{H}\)) and phosphorous (\(\text{P}\)) magnetic resonance spectroscopy (MRS), (2) decreased expression of nuclear genes encoding for proteins involved in mitochondrial energy production on postmortem examination of hippocampal tissue; (3) decreased lymphocytic expression of genes regulating oxidative phosphorylation, and impaired up-regulation of genes encoding for proteins of the electron transport chain after exposure to glucose deprivation; (4) markedly abnormal mitochondrial morphology and distribution on postmortem examination of neurons and glia; and (5) elevated lactate levels in cerebrospinal fluid (CSF).

Consequently, certain bipolar patients might respond poorly to current treatments because mitochondrial dysfunction compromises cerebral energy metabolism. Therefore, treatments that enhance mitochondrial functioning may represent a novel therapeutic approach to bipolar disorder. Acetyl-\(\text{l}\)-carnitine (ALCAR), a naturally occurring mitochondrial metabolite, improves mitochondrial function and energy production in both animals and humans. Moreover, several placebo-controlled trials have found ALCAR efficacious in various depressive spectrum disorders, making it an intriguing candidate treatment for the depressed phase of bipolar disorder.

However, although ALCAR may increase energy production, it may also increase production of reactive oxygen species—damaging mitochondrial DNA, proteins, and lipids, and thus further exacerbating defects in energy production. \(\alpha\)-Lipoic acid (ALA), a mitochondrial coenzyme, is a potent antioxidant, and thus an ideal companion agent with ALCAR to increase mitochondrial metabolic activity without increasing oxidative stress. Indeed, animal studies have demonstrated that the ALCAR/ALA combination improves mitochondrial functioning by increasing metabolism and lowering oxidative stress more than either compound alone and combined mitochondrial-enhancing compounds have shown more promise than single agents for the...
treatment of mitochondrial disorders. Both ALCAR and ALA easily cross the blood-brain barrier, possess favorable adverse-effect profiles, and are widely available as over-the-counter supplements, making them especially attractive as potential bipolar disorder treatments.

We hypothesized that ALCAR/ALA would demonstrate significantly greater efficacy than placebo as an augmentation treatment in bipolar depressed patients displaying an incomplete response to conventional treatments. Accordingly, we performed a 12-week placebo-controlled, double-blind, parallel-group, flexible-dose study of ALCAR 1000 to 3000 mg daily plus ALA 600 to 1800 mg daily, added to conventional treatment in depressed bipolar patients.

Additionally, using 31P-MRS, we assessed in vivo changes in mitochondrial functioning by measuring several biological markers of cerebral energy metabolism during treatment. Specifically, we hypothesized that ALCAR/ALA, but not placebo, would increase cerebral intracellular pH (pH) due to decreased lactate production resulting from a reduced dependence on glycolysis for energy production. Our primary brain region of interest was the anterior cingulate cortex (ACC)—a region implicated in the pathophysiology of bipolar disorder. We also performed exploratory analyses of the previously mentioned metabolites and pH across the whole brain and in several other brain regions including the parieto-occipital cortex (POC), frontal cortex, and thalamus.

**MATERIALS AND METHODS**

**Study Design**

We assigned eligible participants to ALCAR/ALA or placebo, in a 1:1 ratio, via a computer-generated randomization schedule. An independent research assistant, not otherwise involved with the study, placed ALCAR and ALA or matching placebo capsules (all obtained from Pure Encapsulations, Sudbury, MA) in numbered bottles, which were assigned sequentially to placebo capsules (all obtained from Pure Encapsulations, Sudbury, MA) in numbered bottles, which were assigned sequentially to study participants at randomization. All participants and study personnel remained blinded to treatment assignments until study termination.

ALCAR/ALA dosing was based on previous clinical trials reporting tolerability and efficacy.\(^{23,36,37}\) Given the lack of experience with these compounds in the treatment of bipolar disorder, we used a flexible-dose design to achieve the highest tolerated doses and hence a maximum chance of biological effect.

A total sample size of 40 was chosen because it had greater than 80% power to detect a 4-point difference between groups if recommended by the participant’s outpatient psychiatrist and judged unlikely to influence depressive symptoms (see later). Participants were permitted to continue as-needed medications as long as the dose and frequency of use did not change significantly during the course of the study.

**Clinical Evaluation**

Participants initially received a screening evaluation, where they signed informed consent for the study, which had been approved by the McLean Hospital Institutional Review Board. We then obtained basic demographic information, medical and psychiatric history, the Structured Clinical Interview for DSM-IV (SCID) to establish the diagnosis of bipolar depression and any other comorbid Axis I disorders, physical examination, vital signs, electrocardiogram, and laboratory tests. We then administered our primary clinical outcome measure, the MADRS, and 3 secondary measures, namely, the 25-item Hamilton Depression Rating Scale (HAM-D), Clinical Global Impression Scale for Severity (CGI-S), and Young Mania Rating Scale (YMRS).

Eligible participants returned in approximately 1 week for a baseline visit to assess adverse events, concomitant medications, vital signs, MADRS, HAM-D, CGI-S, and YMRS. Additionally, those eligible for the MRS component of the study underwent a 31P-MRS scan (detailed later). All participants were then started on either 2 ALCAR (500 mg) capsules and 1 ALA (600 mg) capsule daily or matching placebo, with instructions to take study medication at least 30 minutes before or 60 minutes after eating, because food impairs absorption of ALA.\(^{38}\) Absent dose-limiting adverse effects, ALCAR, and ALA were increased to 1000 mg twice daily and 600 mg twice daily, respectively, at week 1 and to 1000 and 600 mg 3 times daily, respectively, at week 2. Participants unable to tolerate higher doses could reduce to a minimum dose of 1000 and 600 mg daily. Participants were seen at weeks 1, 2, 3, 4, 6, 8, 10, and 12. At each visit, we administered the same outcome measures as at baseline, plus the Clinical Global Impression Scale for Improvement. We also assessed for adverse events and changes in concomitant medications and performed pill counts to assess compliance. Additionally 31P-MRS scans were performed at week 1 and at week 12 for those participating in the MRS component of the study.

**31P-MRS Acquisition**

A dual tuned proton-phosphorus TEM head coil (Bioengineering Inc, Minneapolis, MN) operating at 170.3 MHz for proton and 68.95 MHz for phosphorus was used for all anatomical imaging and spectroscopy. Manual shimming on the unsuppressed global water signal yielded a typical unsuppressed water linewidth of 20 to 30 Hz. A 3-plane scout image set quickly determined the patient’s position within the coil, followed by
high-contrast, T1-weighted sagittal and axial image sets (TE/TR = 6.2/11.4 milliseconds, field of view = 22 x 22 cm, readout duration = 4 milliseconds, receive bandwidth = ±32 kHz, in-plane matrix size = 128 x 256 [sagittal], 256 x 256 [axial], in-plane resolution = 1.90 x 0.94 mm [sagittal], 0.94 x 0.94 mm [axial], axial-plane matrix size = 32 [sagittal], 64 [axial] axial-plane resolution = 2.5 mm [sagittal and axial], scan time = 2 minutes, 30 seconds [sagittal], 5 minutes [axial]) of the entire brain were acquired using a 3-dimensional, magnetization-prepared FLASH imaging sequence (3D-mpFLASH), allowing for clear segmentation between gray matter, white matter, and CSF.

Phosphorous 3-dimensional chemical-shift imaging ($^{31}$P 3D-CSI) used the phosphorus channel of the dual tuned proton-phosphorus head coil. Acquisition parameters were as follows: TR = 500 milliseconds; tip angle = 32 degrees; Rx bandwidth = ±2 kHz; complex points = 1024; readout duration = 256 milliseconds; pre-pulses = 10; pre-acquisition delay = 1.905 milliseconds; field of view (x,y,z) = 330 mm; maximum phase-encode matrix dimension (x,y,z) = 14 x 14 x 14 (zero-filled out to 16 x 16 x 16 before reconstruction). The $^{31}$P 3D-CSI sequence used a spherically bound, sparse-omission,18-20 reduced phase-encoding scheme, with k-space points randomly omitted from the 14 x 14 x 14 matrix in such a way that the degree of k-space point omission gradually increased toward outer k-space. The variable k-space sampling density preserved the sensitivity of the measurement as well as the spatial localization, although greatly reducing scan time.

$^{31}$P-MRS Processing and Analysis

The $^{31}$P 3D-CSI raw data sets were first zero-padded within a 16 x 16 x 16 matrix and each k-space free-induction decay digitally corrected in amplitude, accounting for the discrepancy between theoretical and integer-weighted k-space filter functions. Once spatially resolved, the $^{31}$P 3D-CSI grid was coregistered with the axial T1-weighted images such that the grid was centered midsagitally inside the brain according to anatomical landmarks in both the sagittal and axial planes (Fig. 1). A 4 x 7 x 5 matrix of voxels was centered within the brain so as to exclude voxels adjacent to the temporalis muscle, thus minimizing signal contamination from these muscles. Additionally, voxels too close to the superior and inferior surfaces of the skull were omitted due to low signal-to-noise and susceptibility artifact. Automated software then zero-order phase-corrected each spectrum using the PCr resonance as a navigator and extracted the spatially resolved spectral free-induction decays (time-domain) from each voxel in each scan for separate fitting of each spectrum.

Offline image processing used commercial and custom-written software for tissue segmentation, partial-volume analysis, and grid-shifting. For $^{31}$P 3D-CSI spectral fitting, we used a spectral time-domain fitting program, based on the Marquardt-Levenberg nonlinear, least-squares algorithm, incorporating prior knowledge of spectral peak assignments, chemical-shifts, and J-coupling constants.39 Our spectral model included 10 phosphorous-containing molecules: γ-, α-, and β-NTP; phosphoethanolamine (Ptn), phosphocholine (Pcho), glycerophosphoethanolamine (GPEn), glycerophosphocholine (GPCp), 2,3-diphosphoglycerate (DPG), inorganic phosphate (P), membrane-bound phospholipid (MP), and PCr (Fig. 2). The model assumes Lorentzian lineshape for the singlet PCr and Pi resonances, Lorentzian doublets (1:1) for the γ- and α-NTP resonances and a Lorentzian-modeled triplet structure (1:2:1) for the β-NTP resonance where the NTP J-coupling constant was fixed to 16 Hz. Membrane phospholipid was modeled as a single broad resonance in the phosphodiester region and fixed in chemical shift and linewidth. The dinucleotide (DN) peak was modeled as a Lorentzian singlet fixed in chemical shift and linewidth. The individual constituents for the phosphomonoester (Ptn and Pcho) and phosphodiester (GPEn and GPCho) regions were all modeled as Gaussian singlets because our 4T spectra are coupled and J-coupled dispersion still exists within each one of these phospholipid resonances, thus affecting the lineshape. The linewidth of each resonance as well as the chemical shift of the lower signal-to-noise peaks such as the DPG, Pcho, GPEn, MP, and DN resonances were constrained. Our spectral model is described in more detail elsewhere.39

Estimates of pH were calculated using the chemical shift difference between the resonances for PCr and Pi according to the modified Henderson-Hasselbalch equation.49 A measure of total NTP (α-, β-, and γ-NTP) was also calculated.

Tissue Segmentation and Image Postprocessing

Extracted brain images were then segmented into white matter, gray matter, and CSF using the FSL Brain Extraction
Tool (FMRIB)'s Automated Segmentation Tool (FAST). We then determined the contributions of tissue type (gray or white matter) and CSF in each voxel. In this process, we convolved the mathematically modeled, 3-dimensional point-spread function (3D-PSF) from the sparse k-space sampling scheme, digitally sampled in a \(256 \times 256 \times 64\) matrix, with the coregistered binary images (also digital matrices of \(256 \times 256 \times 64\)) to obtain theoretically correct pixel counts of the contribution of each tissue type to each voxel based on the 3D-PSF weighted distribution.

**Statistical Analysis**

Baseline group characteristics were compared by Fisher exact tests for categorical variables and 2-sample \(t\) tests for continuous variables.

The primary efficacy analysis was a longitudinal random regression analysis comparing the rate of change of the outcome variables (MADRS, HAM-D, CGI-S, and YMRS) during the treatment period between groups. We used a model for the mean of the outcome variable that included terms for treatment, time (as a continuous variable), and treatment-by-time interaction. The coefficient for the treatment-by-time term quantifies the rate of clinical improvement, which we expressed as the estimated change in the measure at week 12. We also conducted a secondary analysis comparing change from baseline to end point (using last observation carried forward) between groups using the 2-sample \(t\) test.

We compared the groups on \(^{31}\text{P}-\text{MRS}\) measures using a similar longitudinal random regression model with terms for the \(^{31}\text{P}-\text{MRS}\) measure, time (modeled as a categorical variable), and treatment-by-time interaction. The coefficient for the treatment-by-time term quantifies the rate of change in these metabolites at week 1 and week 12, adjusted for baseline values. We first assessed whether there was a significant treatment-by-time interaction (2 degrees of freedom \(\chi^2\) test), and if there was, we then examined the coefficients for the individual interaction terms to determine whether they were statistically significant.

For all longitudinal analyses, we used generalized estimating equations to adjust standard errors to account for the correlation of observations within individuals, with the working covariance structure being first-order autoregressive for the models where time was a continuous variable and independence for the models where time was a categorical variable.

We used linear regression to assess associations between improvement in depressive symptoms (change in MADRS from baseline using LOCF) and baseline levels of \(^{31}\text{P}-\text{MRS}\) metabolites and pH, as well as change in these metabolites at week 1 and week 12 in each brain region (ACC, POC, frontal cortex, thalamus, and whole brain).

All analyses were performed using Stata 9.2 software. \(\alpha\) was set at 0.05, 2-tailed.

**RESULTS**

**Clinical Trial Analyses**

**Participant Characteristics and Study Flow**

Sixty-eight participants signed informed consent for the study from September 4, 2008, to January 25, 2011. Of these, 28 were withdrawn from the study before receiving treatment (lost to follow-up \(n = 14\); did not meet inclusion criteria \(n = 7\); met exclusion criteria \(n = 5\); consent withdrawn \(n = 2\)). Forty were randomized to treatment and all received at least 1 postbaseline assessment. Of the 20 participants randomized to each study arm, 11 (55%) in the ALCAR/ALA group and 14 (70%) in the placebo group completed study procedures. Reasons for discontinuation included adverse event \(n = 5\), noncompliance with study procedures \(n = 3\), and consent withdrawn \(n = 2\). Forty were randomized to treatment and all received at least 1 postbaseline assessment. Of the 20 participants randomized to each study arm, 11 (55%) in the ALCAR/ALA group and 14 (70%) in the placebo group completed study procedures. Reasons for discontinuation included adverse event \(n = 5\), noncompliance with study procedures \(n = 3\), and consent withdrawn \(n = 2\). Forty were randomized to treatment and all received at least 1 postbaseline assessment. Of the 20 participants randomized to each study arm, 11 (55%) in the ALCAR/ALA group and 14 (70%) in the placebo group completed study procedures. Reasons for discontinuation included adverse event \(n = 5\), noncompliance with study procedures \(n = 3\), and consent withdrawn \(n = 2\). Forty were randomized to treatment and all received at least 1 postbaseline assessment. Of the 20 participants randomized to each study arm, 11 (55%) in the ALCAR/ALA group and 14 (70%) in the placebo group completed study procedures. Reasons for discontinuation included adverse event \(n = 5\), noncompliance with study procedures \(n = 3\), and consent withdrawn \(n = 2\). Forty were randomized to treatment and all received at least 1 postbaseline assessment. Of the 20 participants randomized to each study arm, 11 (55%) in the ALCAR/ALA group and 14 (70%) in the placebo group completed study procedures. Reasons for discontinuation included adverse event \(n = 5\), noncompliance with study procedures \(n = 3\), and consent withdrawn \(n = 2\). Forty were randomized to treatment and all received at least 1 postbaseline assessment. Of the 20 participants randomized to each study arm, 11 (55%) in the ALCAR/ALA group and 14 (70%) in the placebo group completed study procedures. Reasons for discontinuation included adverse event \(n = 5\), noncompliance with study procedures \(n = 3\), and consent withdrawn \(n = 2\). Forty were randomized to treatment and all received at least 1 postbaseline assessment. Of the 20 participants randomized to each study arm, 11 (55%) in the ALCAR/ALA group and 14 (70%) in the placebo group completed study procedures. Reasons for discontinuation included adverse event \(n = 5\), noncompliance with study procedures \(n = 3\), and consent withdrawn \(n = 2\).
Mean (SD) daily doses of ALCAR and ALA at end point were 2275 (750.6) and 1365 (450.4) mg, respectively. Three participants were permitted to alter existing medications during the study. One, receiving ALCAR/ALA, was permitted to increase citalopram from 20 to 40 mg daily at week 1. This participant withdrew at week 6 due to worsening depressive symptoms. Another, receiving placebo, was permitted to increase quetiapine from 100 to 150 mg at bedtime at week 2. This participant withdrew at week 4. Another, receiving ALCAR/ALA, was permitted to switch at week 6 from eszopiclone 3 mg to zolpidem 10 mg at bedtime due to a change in prescription drug coverage.

### Efficacy Analyses

Mean MADRS scores for each treatment group are presented in Figure 3. Neither the primary longitudinal analysis nor the end point analysis showed significant differences between ALCAR/ALA and placebo on any outcome measure (Table 2).

### Adverse Events

Adverse events were generally minor. The most frequently reported adverse events in the ALCAR/ALA group versus the placebo group included diarrhea (30% ALCAR/ALA vs 15% placebo), foul-smelling urine (25% vs 5%), rash (20% vs 0%), constipation (15% vs 5%), and dyspepsia (15% vs 0%). There were 2 serious events. One participant was withdrawn from the study when hospitalized for acute chest pain; cardiac evaluation proved unremarkable and the participant was found to be taking placebo. Another participant, receiving ALCAR/ALA, developed abdominal pain, found due to small bowel obstruction, 31 days after completing study medication. Given this time interval, the event was judged unrelated to study treatment. Additionally, 1 participant receiving ALCAR/ALA displayed mildly elevated liver function tests at study completion, felt likely due to concomitant treatment with valproate.

### \(^{31}\)P-MRS Analyses

Twenty participants (10 receiving ALCAR/ALA, 10 receiving placebo) participated in the imaging component of the study. Because of attrition, only 12 of these (5 ALCAR/ALA and 7 placebo) yielded complete imaging data sets. We found

![FIGURE 3. The mean scores on the MADRS over 12 weeks of treatment with ALCAR plus ALA or placebo. Error bars represent standard error.](image-url)
TABLE 2. Values of Outcome Measures After 12 Weeks of Treatment (Completers and LOCF) and Model-Based Estimates of Differences Between the Groups

<table>
<thead>
<tr>
<th>Outcome Measure</th>
<th>Completers</th>
<th>Placebo</th>
<th>LOCF</th>
<th>Placebo</th>
<th>Longitudinal Analysis, Mean 12-wk Change</th>
<th>End Point Analysis, Mean 12-wk Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALCAR/ALA</td>
<td>Placebo</td>
<td>ALCAR/ALA</td>
<td>Placebo</td>
<td>Estimate (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>MADRS score</td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td>-1.4 (-6.2 to 3.4)</td>
<td>0.58</td>
</tr>
<tr>
<td>HAM-D score</td>
<td>17.8 (5.6)</td>
<td>17.9 (5.9)</td>
<td>17.5 (6.4)</td>
<td>18.5 (6.2)</td>
<td>-0.9 (-5.3 to 3.4)</td>
<td>0.92</td>
</tr>
<tr>
<td>YMRS score</td>
<td>1.4 (2.2)</td>
<td>2.1 (1.9)</td>
<td>1.7 (2.2)</td>
<td>2.1 (1.8)</td>
<td>-0.4 (-1.9 to 1.0)</td>
<td>0.58</td>
</tr>
<tr>
<td>CGI-S score</td>
<td>4 (0.8)</td>
<td>4.1 (0.7)</td>
<td>4 (0.9)</td>
<td>4.2 (0.7)</td>
<td>-0.03 (-0.56 to 0.50)</td>
<td>0.92</td>
</tr>
</tbody>
</table>

CI indicates confidence interval.

TABLE 3. Mean (SD) Metabolite and pH Levels by Region*†

<table>
<thead>
<tr>
<th></th>
<th>PCr Mean (SD)</th>
<th>ALCAR/ALA</th>
<th>Placebo</th>
<th>β-NTP Mean (SD)</th>
<th>ALCAR/ALA</th>
<th>Placebo</th>
<th>pH Mean (SD)</th>
<th>ALCAR/ALA</th>
<th>Placebo</th>
<th>Total NTP Mean (SD)</th>
<th>ALCAR/ALA</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACC</td>
<td>0.177 (0.037)</td>
<td>0.156 (0.008)</td>
<td>0.07 (0.01)</td>
<td>0.08 (0.023)</td>
<td>6.99 (0.046)</td>
<td>7.03 (0.057)</td>
<td>0.244 (0.025)</td>
<td>0.252 (0.03)</td>
<td>0.230 (0.009)</td>
<td>0.233 (0.009)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>POC</td>
<td>0.167 (0.011)</td>
<td>0.168 (0.002)</td>
<td>0.05 (0.006)</td>
<td>0.067 (0.011)</td>
<td>7.02 (0.036)</td>
<td>7.03 (0.017)</td>
<td>0.237 (0.009)</td>
<td>0.234 (0.025)</td>
<td>0.234 (0.025)</td>
<td>0.234 (0.025)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>0.174 (0.022)</td>
<td>0.183 (0.038)</td>
<td>0.072 (0.015)</td>
<td>0.067 (0.016)</td>
<td>7.0 (0.022)</td>
<td>6.99 (0.031)</td>
<td>0.225 (0.025)</td>
<td>0.212 (0.017)</td>
<td>0.235 (0.014)</td>
<td>0.225 (0.021)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thalamus</td>
<td>0.167 (0.022)</td>
<td>0.16 (0.016)</td>
<td>0.066 (0.005)</td>
<td>0.06 (0.02)</td>
<td>7.02 (0.024)</td>
<td>7.01 (0.021)</td>
<td>0.235 (0.014)</td>
<td>0.225 (0.021)</td>
<td>0.235 (0.014)</td>
<td>0.225 (0.021)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole brain</td>
<td>0.169 (0.014)</td>
<td>0.169 (0.012)</td>
<td>0.064 (0.004)</td>
<td>0.064 (0.005)</td>
<td>6.99 (0.007)</td>
<td>6.99 (0.008)</td>
<td>0.229 (0.011)</td>
<td>0.225 (0.008)</td>
<td>0.225 (0.008)</td>
<td>0.225 (0.008)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACC</td>
<td>0.169 (0.002)</td>
<td>0.201 (0.041)</td>
<td>0.073 (0.001)</td>
<td>0.072 (0.006)</td>
<td>6.95 (0.063)</td>
<td>7.0 (0.128)</td>
<td>0.245 (0.069)</td>
<td>0.246 (0.036)</td>
<td>0.234 (0.036)</td>
<td>0.234 (0.036)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>POC</td>
<td>0.149 (0.005)</td>
<td>0.17 (0.015)</td>
<td>0.066 (0.008)</td>
<td>0.066 (0.011)</td>
<td>7.01 (0.013)</td>
<td>7.03 (0.032)</td>
<td>0.234 (0.024)</td>
<td>0.233 (0.027)</td>
<td>0.234 (0.027)</td>
<td>0.234 (0.027)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>0.186 (0.028)</td>
<td>0.186 (0.052)</td>
<td>0.065 (0.005)</td>
<td>0.066 (0.01)</td>
<td>6.99 (0.023)</td>
<td>6.97 (0.021)</td>
<td>0.229 (0.02)</td>
<td>0.23 (0.013)</td>
<td>0.235 (0.005)</td>
<td>0.234 (0.013)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thalamus</td>
<td>0.162 (0.02)</td>
<td>0.165 (0.015)</td>
<td>0.064 (0.014)</td>
<td>0.068 (0.003)</td>
<td>6.92 (0.218)</td>
<td>7.0 (0.035)</td>
<td>0.237 (0.005)</td>
<td>0.242 (0.013)</td>
<td>0.235 (0.005)</td>
<td>0.235 (0.005)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole brain</td>
<td>0.163 (0.014)</td>
<td>0.17 (0.013)</td>
<td>0.064 (0.002)</td>
<td>0.065 (0.004)</td>
<td>6.99 (0.01)</td>
<td>6.99 (0.007)</td>
<td>0.229 (0.006)</td>
<td>0.232 (0.005)</td>
<td>0.232 (0.005)</td>
<td>0.232 (0.005)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*There were no significant group differences for change in metabolite or pH levels from baseline (ie, significant treatment-by-time interactions in our model assessed by 2 degrees of freedom x² test [see text]), except for (1) PCr in the ACC (P = 0.02), but analysis of the individual interaction terms showed that levels at neither time point were significantly different from baseline; and (2) PCr in the POC (P = 0.002), with univariate analysis showing that week 12 was significantly decreased from baseline (P = 0.003).

†Metabolite levels are measured in arbitrary units.
creatinine monohydrate, might have more potent effects on mitochondrial functioning and hence greater antidepressant efficacy. For instance, Berk et al. found that NAC (which increases synthesis of the potent antioxidant glutathione), added to treatment-as-usual in bipolar patients, significantly decreased depressive symptoms and improved quality of life compared to placebo. However, these investigators administered NAC for 24 weeks—twice the duration of our study—and found a significant NAC-placebo difference only after week 20, suggesting that mitochondrial supplements might require more time to yield clinical benefit. Moreover, although we enrolled only depressed bipolar patients, Berk et al. examined NAC as a maintenance treatment, enrolling bipolar patients irrespective of current mood state. Therefore, mitochondrial enhancers such as NAC might be best, not as treatments of acute mood episodes, but as add-on maintenance therapies to manage difficult-to-treat subsyndromal symptoms—an area of great clinical need. However, a recent open-label study by the same group found a significant reduction in depressive symptoms after only 8 weeks of NAC treatment in patients with bipolar depression. Importantly, in addition to its effects on glutathione synthesis, NAC affects glutamatergic neurotransmission, which has also been implicated in the pathophysiology of bipolar disorder. Therefore, further investigation into the exact mechanism(s) of action of NAC is warranted. Recently, an open-label study of CoQ10 in geriatric patients with bipolar depression demonstrated antidepressant effects early in treatment, which dissipated by the end of the 8-week study, supporting further investigation in larger controlled trials.

We were surprised to find little effect of ALCAR/ALA on 31P-MRS markers of cerebral energy metabolism. ALCAR is thought to exert many of its biological effects through the action of its carnitine and acyl moieties. Specifically, carnitine is important in the transport of fatty acids into mitochondria to undergo β-oxidation—an important source of mitochondrial energy production—and acylcarnitines, when oxidized within mitochondria, release energy and form acetyl-CoA, which enters the tricarboxylic acid cycle. Through these mechanisms, ALCAR is hypothesized to boost mitochondrial efficiency, increasing ATP production. Accordingly, adult and aged rats administered ALCAR show both an increase in ATP and PCr as measured by 31P nuclear magnetic spectroscopy. However, our imaging analyses yield only 2 significant associations (see above)—and these likely represented chance findings, given the number of comparisons made and the fact that both were counter to the hypothesized direction of change for these metabolites.

Several hypotheses might explain our negative findings. First, the small imaging sample size (only 12 participants yielded complete 31P-MRS data sets) increases the likelihood for type II error. Second, the dose of ALCAR/ALA may have been inadequate to achieve the desired biological effect—particularly at week 1 when participants were only taking 1000 mg/600 mg daily. Third, as mentioned previously, 12 weeks of ALCAR/ALA treatment may have been too brief to benefit mitochondrial functioning. Fourth, lacking 31P-MRS data from a control group, we could not calibrate the extent of mitochondrial dysfunction in our study population, and hence cannot exclude a ceiling effect. In other words, our participants who contributed imaging data may have had normal PCr and β-NTP levels to begin with, and thus showed minimal increase in PCr and β-NTP levels with ALCAR/ALA treatment. Fifth, in contrast to animal data, the effects of ALCAR/ALA on human mitochondrial functioning may be too weak to significantly impact the neuroimaging markers of cerebral energy metabolism used in our study. Indeed, little is known about the effects of ALCAR/ALA on cerebral energy metabolism in humans; to our knowledge, the only other in vivo study besides ours examined 2 patients with geriatric depression. This study found improvement in depression after 12 weeks of ALCAR 3 g/d, and improvement was associated with increased levels of PCr in the prefrontal cortex as measured by 31P-MRS. However, geriatric depression may respond differently to ALCAR, given the effects of aging on mitochondrial functioning and cerebral bioenergetics.

It is important to note several limitations of this study. First, the sample size was small, limiting statistical power. Second, we did not place restrictions on concomitant medications during the course of the study. Although this approach increases the potential generalizability of our results, it runs the risk of obscuring between-group differences. Third, we enrolled patients with both type I and type II bipolar disorder, increasing the biological heterogeneity of our sample. Although we found no evidence for differences in efficacy based on bipolar type in our study, it is theoretically plausible that mitochondrial dysfunction may be more prevalent in a particular subtype of bipolar disorder. Therefore, a more homogenous sample of bipolar patients could be more biologically predisposed to benefit from ALCAR/ALA treatment. Fourth, as discussed previously, we did not include a non–bipolar disorder comparison group in the imaging study, making it difficult to assess the degree of mitochondrial dysfunction at baseline in our study cohort. Fifth, evidence suggests that intravenous administration of ALCAR may be necessary to achieve antidepressant effects given its nonlinear pharmacokinetics and low absolute bioavailability. Therefore, it is possible that oral ALCAR/ALA, as administered in this study, did not result in sufficient brain concentrations to significantly impact cerebral mitochondrial functioning.

In summary, although our findings may discourage further investigation of ALCAR/ALA for treatment of bipolar depression, they should not discourage the study of other potentially beneficial mitochondrial-enhancing agents for bipolar disorder—especially maintenance treatment to address subthreshold mood symptoms. Although substantial evidence suggests a role for mitochondrial dysfunction in the pathophysiology of bipolar disorder, the cause of this dysfunction remains obscure, making it difficult to develop targeted treatments. Furthermore, few drug treatments have been developed to enhance mitochondrial functioning through specific biological pathways. As a result, most available mitochondrial-modulating compounds are low-potency over-the-counter supplements with inexact mechanisms of action. Nevertheless, emerging findings suggest that at least one of these mitochondrial enhancers, NAC, may have potential use in depressed bipolar patients.

In short, despite the negative results of the present study, it seems important to pursue the specific molecular underpinnings of mitochondrial dysfunction in bipolar disorder, and to develop more targeted and potent mitochondrial enhancers. Progress in this area may create new opportunities for treatment of this serious and often refractory condition.

ACKNOWLEDGMENT

The authors thank Pure Encapsulations for providing ALCAR, ALA, and matching placebo capsules for this study (note: Pure Encapsulations provided no additional financial support for this study and had no input into the design or analysis of this study).

AUTHOR DISCLOSURE INFORMATION

B.P.B. has received research grant support from Eli Lilly. J.I.H. has received research grant support from Eli Lilly.
Ortho-McNeil Janssen Scientific Affairs, and Otsuka, and has been a consultant for Alkermes, Eli Lilly, Pfizer, Roche, and Shire. H.G.P. has received research grant support from Solvay Pharmaceuticals. P.E.R. has received research grant support from GlaxoSmithKline and Roche, has been a consultant to Novartis, Roche, Ridge Diagnostics, and Kyowa Hakko Kirin, owns stock in Ridge Diagnostics, and has received royalty payments from Repligen. None of the other authors reported any biomedical financial interests or potential conflicts of interest.

REFERENCES


www.psychopharmacology.com

© 2013 Lippincott Williams & Wilkins


Effect of the probiotic *Saccharomyces boulardii* on cholesterol and lipoprotein particles in hypercholesterolemic adults: A single-arm, open-label pilot study.  
Effect of the probiotic *Saccharomyces boulardii* on cholesterol and lipoprotein particles in hypercholesterolemic adults:
A single-arm, open-label pilot study.
Effect of the Probiotic *Saccharomyces boulardii* on Cholesterol and Lipoprotein Particles in Hypercholesterolemic Adults: A Single-Arm, Open-Label Pilot Study

Jennifer Joan Ryan, ND, MS, Douglas Allen Hanes, PhD, Morgan Beth Schafer, MA, Jeremy Mikolai, ND, and Heather Zwickey, PhD

Abstract

**Objectives:** Elevated blood cholesterol levels are a major risk factor for coronary artery disease, the leading cause of death worldwide. Probiotics have been investigated as potential cholesterol-lowering therapies, but no previous studies have assessed the effect of the probiotic yeast *Saccharomyces boulardii* on cholesterol levels in human volunteers. The objective of this study was to examine the effect of *S. boulardii* on serum cholesterol and lipoprotein particles in hypercholesterolemic adults.

**Design:** This study was a single-arm, open-label pilot study.

**Subjects:** Twelve hypercholesterolemic participants were recruited into the study; one dropped out.

**Intervention:** Participants took $5.6 \times 10^{10}$ colony forming unit (CFU) encapsulated *S. boulardii* (*Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079) twice daily for an 8-week period.

**Outcome measures:** Fasting concentrations of cholesterol (total cholesterol, low-density lipoprotein-cholesterol [LDL-C], high-density lipoprotein-cholesterol [HDL-C], and triglycerides), lipoprotein particles (very-low-density lipoprotein-particle [VLDL-P], remnant lipoprotein particle [RLP-P], total LDL-P, LDL III-P, LDL IV-P, total HDL-P, and HDL 2b-P), and additional cardiovascular biomarkers (apo B-100, lipoprotein [a], high-sensitivity C-reactive protein, homocysteine, fibrinogen, and insulin) were measured at baseline, after 4 weeks, and after 8 weeks.

**Results:** Remnant lipoprotein particles decreased by 15.5% ($p = 0.03$) over the 8-week period. The remaining outcome measures were not significantly altered.

**Conclusions:** In this pilot study, 8 weeks of daily supplementation with *S. boulardii* lowered remnant lipoprotein, a predictive biomarker and potential therapeutic target in the treatment and prevention of coronary artery disease.

**Introduction**

Coronary artery disease (CAD) is the leading cause of death worldwide. Elevated blood cholesterol levels are a major risk factor for CAD, and standard treatment options for elevated cholesterol include pharmacotherapy, and nutritional and lifestyle modifications. Probiotics in capsule form and fermented dairy products containing live probiotics have also been investigated as potential cholesterol-lowering therapies. Promising results have included significant reductions in total cholesterol and low-density lipoprotein cholesterol (LDL-C), as well as increases in high-density lipoprotein cholesterol (HDL-C). Various mechanisms of lipid lowering by probiotics have been proposed, including the assimilation and incorporation of cholesterol into bacterial cellular membranes, the binding of cholesterol to bacterial cellular surfaces, and the deconjugation of intestinal bile salts by bacterial bile salt hydro-lase. In *vitro* studies have shown that several strains of *Lactobacillus* and *Lactococcus* can remove cholesterol from culture medium. Similar to bacterial strains, the yeasts *Saccharomyces cerevisiae* and *Saccharomyces boulardii* can remove cholesterol from laboratory culture medium.

*S. boulardii* is a probiotic and substrain of the more well-known budding yeast *S. cerevisiae*. Like many other probiotics, *S. boulardii* has been investigated as a treatment for several acute and chronic gastrointestinal diseases. A recent meta-analysis showed that it effectively prevents antibiotic-associated diarrhea and traveler’s diarrhea.

Previous human subject investigations that have examined the potential cholesterol-lowering ability of probiotics have focused on bacterial rather than yeast strains, and no
previous studies have examined the effect of *S. boulardii* on cholesterol levels in human volunteers. However, a study in mice showed that animals given feed that had been fermented with *S. boulardii* and *L. casei* had significantly lower levels of total and LDL cholesterol than mice given control feed without these probiotics.14

To our knowledge, previous investigations of the effect of probiotics on cholesterol have not investigated their effect on lipoprotein particles. Similar to standard cholesterol levels, lipoprotein particle levels correlate strongly with the risk of future cardiovascular events.15 Additionally, some individuals exhibit discordance between cholesterol levels and lipoprotein particle numbers, and lipid-lowering therapies may alter cholesterol levels differently than they alter lipoprotein particles. Therefore, monitoring lipoprotein particles in addition to standard cholesterol levels may provide more extensive assessment and monitoring of cardiovascular disease risk than measuring standard cholesterol levels alone.15–17

The current study aimed to collect preliminary evidence on the effect of the probiotic *S. boulardii* on lipids, lipoprotein particles, and additional cardiovascular biomarkers in hypercholesterolemic adults. As a pilot investigation, this study also aimed to assess the feasibility of the study methods.

**Materials and Methods**

**Subjects**

Otherwise healthy adults aged 21–69 years with total cholesterol of 200–275 mg/dL, HDLC of <70 mg/dL, and body mass index (BMI) of 20–45 kg/m² were recruited online and via flyers posted on bulletin boards in the Portland, OR, area. The recruitment target was 12 participants. Exclusion criteria were as follows: a previous cardiovascular event, congestive heart failure, pacemaker, arrhythmia, valvular disease, or heart surgery (after the age of 1); history of rheumatic fever or abnormal echocardiogram; family history of premature CAD; diabetes, immunodeficiency disorder, having a central venous catheter, bowel, hepatic, or renal disease, untreated hypothyroidism, cancer within the last 5 years, pregnancy, lactation, or planning pregnancy. Participants were also excluded if they were taking cholesterol-lowering prescription medication, red yeast rice, plant sterols, policosanols, fish oil >2000 mg/day, niacin >500 mg/day, CoQ10 >200 mg/day, encapsulated garlic >6 g/day, *Commiphora mukul* >800 mg/day, probiotic supplements, or systemic antifungal, corticosteroid, or immunosuppressant medications. Furthermore, participants were excluded if they were planning to make significant dietary or lifestyle changes during the study period or if their blood pressure was >160 mmHg (systolic) or >100 mmHg (diastolic) upon screening. The study protocol was approved by the Institutional Review Board at National College of Natural Medicine. All volunteers provided written informed consent before participation in the study.

**Study design**

The study was a single-arm, open-label pilot study with *S. boulardii* supplementation for 8 weeks. Participants were screened over the phone and at an in-person screening visit. Data were collected from qualifying participants at baseline, after 4 weeks, and after 8 weeks of treatment. Eligible participants were instructed to take four *S. boulardii* capsules with food twice per day. The supplement contained *S. boulardii* (*Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079) and rice starch in a cellulose capsule (Pure Encapsulations, Inc., Sudbury, MA); each capsule contained 1.4×10¹⁰ colony forming unit (CFU) per capsule at the study end point as measured through third-party analysis (Exova, Portland, OR). Participants were asked to maintain their usual diet and level of physical activity throughout the study.

**Data collection**

Blood pressure was measured at the screening visit. Height and weight were obtained for BMI calculation at baseline and at the end of the study. Physical activity level was assessed at each study visit by asking participants to rate their average weekly exercise level over the previous 4 weeks; they were provided with categorical responses in 1.5-hour increments. Blood samples were obtained by venipuncture at the screening, baseline, week 4, and week 8 visits. Participants were asked to fast for 12 hours and to abstain from alcohol for 24 hours before blood draws. Supplement compliance was monitored by daily documentation on study calendars given to the participants and an end-of-study count of returned capsules.

**Sample analysis**

Serum collected at the screening visit was analyzed for total cholesterol and HDL-C levels by Quest Diagnostics (Seattle, WA) on an Olympus AU5400 analyzer using reagent kits (Beckman Coulter, Brea, CA, for total cholesterol, and Roche Diagnostics, Indianapolis, IN, for HDL-C). Plasma collected at the baseline, week 4, and week 8 visits was analyzed for fibrinogen levels by Quest Diagnostics (San Juan Capistrano, CA) on a Siemens BCS-XP analyzer using Dade Thrombin Reagent (Siemens, Munich, Germany). Serum collected at the baseline, week 4, and week 8 visits was analyzed for total cholesterol, LDL-C, HDL-C, triglycerides, lipoprotein particles (very-low-density lipoprotein particle [VLDL-P], remnant lipoprotein particle [RLP-P], total LDL-P, LDL-3-P, LDL-4-P, total HDL-P, HDL2b-P, and non-HDL-P), apolipoprotein B-100 (apo B-100), lipoprotein(a) (Lp[a]), high-sensitivity C-reactive protein (hs-CRP), insulin, and homocysteine by SpectraCell Laboratories (Houston, TX) using patented technology including analytical ultracentrifugation.18

**Statistical analysis**

All statistical analyses were performed using SPSS v.20 software (IBM Corp., Armonk, NY). Data for one participant were not available at the week 8 time point; values of all variables at week 8, for this participant, were imputed using the “last observation carried forward” method, according to study protocol. Five (of 33) measurements of insulin, from two participants, were below the laboratory detection threshold; for analysis, they were set equal to the threshold value of 4 μIU/mL. Similarly, 10 (of 33) measurements of Lp(a), from 4 participants, were set equal to the lower Lp(a) threshold of 5 mg/dL.

As primary results of this pilot study, means and standard deviations of all outcomes were calculated, at each of the baseline, week 4, and week 8 time points. Since week 8 changes are considered primary, baseline-to-week 8 changes and percent change relative to baseline were determined.
Table 1. Participant Characteristics at Baseline

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>% or mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian (%)</td>
<td>90.91%</td>
</tr>
<tr>
<td>Two or more races (%)</td>
<td>9.09%</td>
</tr>
<tr>
<td>Male (%)</td>
<td>90.91%</td>
</tr>
<tr>
<td>Female (%)</td>
<td>9.09%</td>
</tr>
<tr>
<td>Age (years)</td>
<td>38.27 (8.52)</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>85.17 (10.99)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>28.02 (5.36)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>108.45 (12.92)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>70.96 (10.02)</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>229.73 (29.92)</td>
</tr>
<tr>
<td>Non-HDL cholesterol (mg/dL)</td>
<td>181.73</td>
</tr>
<tr>
<td>Total HDL cholesterol (mg/dL)</td>
<td>48.00</td>
</tr>
<tr>
<td>Total LDL cholesterol (mg/dL)</td>
<td>167.82</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>167.82 (25.31)</td>
</tr>
<tr>
<td>Non-HDL-P (nmol/L)</td>
<td>1125.73</td>
</tr>
<tr>
<td>Total HDL-P (nmol/L)</td>
<td>7893.09</td>
</tr>
<tr>
<td>Total LDL-P (nmol/L)</td>
<td>1043.82</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>107.84 (13.36)</td>
</tr>
<tr>
<td>LDL-3-P (nmol/L)</td>
<td>13.68</td>
</tr>
<tr>
<td>LDL-4-P (nmol/L)</td>
<td>86.01</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>142.45</td>
</tr>
<tr>
<td>TC/HDLC (mg/dL)</td>
<td>9.82</td>
</tr>
<tr>
<td>TC/HDL-C</td>
<td>34.82</td>
</tr>
<tr>
<td>RLP-P (nmol/L)</td>
<td>24.74</td>
</tr>
<tr>
<td>VLDL-P (nmol/L)</td>
<td>1043.82</td>
</tr>
<tr>
<td>HDL-2b-P (nmol/L)</td>
<td>1639.18</td>
</tr>
<tr>
<td>Non-HDL-P (nmol/L)</td>
<td>1125.73</td>
</tr>
<tr>
<td>Non-HDL-C (mg/dL)</td>
<td>181.73</td>
</tr>
</tbody>
</table>

Results

Subject characteristics

One hundred individuals responded to advertisements and 89 phone screenings were completed, with 52% eligibility. Thirty-six screening visits were completed with 39% eligibility. Twelve participants enrolled in the study, 11 completed the study as part of the intention-to-treat population, and 1 dropped out of the study shortly after the baseline visit because of an acute illness. Additional recruitment and eligibility details are presented in Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/acm. Characteristics of the 11 participants in the intention-to-treat population are presented in Table 1.

The study population consisted of nonsmoking predominantly Caucasian males who were normotensive and hypercholesterolemic. Based on BMI, 27.3% of participants were of normal weight, 54.5% were overweight, and 18.2% were obese. Two participants were taking stable dosages of fish oil (1000 and 1600 mg daily) and two other participants were taking stable dosages of niacin (20 mg niacinamide in a daily multiple vitamin) within allowable amounts (as per exclusion criteria) during the study; each of these four participants had been taking the supplements before beginning the study for at least six months. None of the participants were taking CoQ10, encapsulated garlic, or Commiphora mukul. Supplement compliance, as monitored by an end-of-study count of returned capsules, averaged 92.1% (with a range of 76.1–100.0%).

Serum lipids and lipoprotein particles

Means and standard deviations of lipids and lipoprotein particles at each study time point are presented in Table 2. Levels of total cholesterol, LDL-C, HDL-C, and triglycerides were unchanged throughout the 8-week study (Fig. 1). VLDL-

Table 2. Lipids and Lipoprotein Particles at Baseline, After 4 Weeks, and After 8 Weeks

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Baseline</th>
<th>Week 4</th>
<th>Week 8</th>
<th>p</th>
<th>Δ</th>
<th>% Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dL)</td>
<td>229.73 ± 29.92</td>
<td>224.64 ± 39.27</td>
<td>224.18 ± 27.61</td>
<td>0.73</td>
<td>-5.55 ± 36.28</td>
<td>-1.72 ± 11.30</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>167.82 ± 25.31</td>
<td>168.55 ± 36.74</td>
<td>166.36 ± 30.85</td>
<td>0.96</td>
<td>-1.45 ± 24.74</td>
<td>-0.28 ± 14.24</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>48.00 ± 6.03</td>
<td>47.18 ± 7.14</td>
<td>46.09 ± 4.76</td>
<td>0.50</td>
<td>-1.91 ± 4.04</td>
<td>-3.41 ± 8.88</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>142.45 ± 77.09</td>
<td>117.00 ± 40.42</td>
<td>132.64 ± 86.01</td>
<td>0.33</td>
<td>9.82 ± 34.41</td>
<td>6.01 ± 24.17</td>
</tr>
<tr>
<td>TC/HDLC</td>
<td>4.84 ± 0.77</td>
<td>4.79 ± 0.72</td>
<td>4.93 ± 0.90</td>
<td>0.69</td>
<td>0.09 ± 0.52</td>
<td>2.03 ± 10.74</td>
</tr>
<tr>
<td>Non-HDL-C (mg/dL)</td>
<td>181.73 ± 28.91</td>
<td>177.45 ± 35.53</td>
<td>178.09 ± 29.39</td>
<td>0.81</td>
<td>-3.64 ± 24.45</td>
<td>-1.19 ± 13.53</td>
</tr>
<tr>
<td>VLDL-P (nmol/L)</td>
<td>81.73 ± 46.14</td>
<td>69.55 ± 31.25</td>
<td>68.36 ± 43.37</td>
<td>0.29</td>
<td>-13.36 ± 24.11</td>
<td>-13.40 ± 33.94</td>
</tr>
<tr>
<td>RLP-P (nmol/L)</td>
<td>136.91 ± 48.29</td>
<td>124.09 ± 47.57</td>
<td>110.82 ± 41.66</td>
<td>0.03</td>
<td>-26.09 ± 34.82</td>
<td>-15.46 ± 30.12</td>
</tr>
<tr>
<td>Total LDL-P (nmol/L)</td>
<td>1043.82 ± 154.58</td>
<td>1043.73 ± 211.74</td>
<td>1000.91 ± 121.10</td>
<td>0.53</td>
<td>-42.91 ± 134.57</td>
<td>-3.01 ± 12.66</td>
</tr>
<tr>
<td>LDL-3-P (nmol/L)</td>
<td>368.18 ± 119.29</td>
<td>397.36 ± 156.08</td>
<td>393.27 ± 107.84</td>
<td>0.73</td>
<td>25.09 ± 110.86</td>
<td>11.47 ± 31.71</td>
</tr>
<tr>
<td>LDL-4-P (nmol/L)</td>
<td>81.64 ± 13.68</td>
<td>76.91 ± 18.62</td>
<td>89.73 ± 18.35</td>
<td>0.08</td>
<td>8.09 ± 20.08</td>
<td>12.07 ± 26.65</td>
</tr>
<tr>
<td>HDL-2b-P (nmol/L)</td>
<td>1639.18 ± 239.59</td>
<td>1603.09 ± 281.80</td>
<td>1644.82 ± 204.48</td>
<td>0.82</td>
<td>5.64 ± 196.41</td>
<td>1.52 ± 13.57</td>
</tr>
<tr>
<td>Non-HDL-P (nmol/L)</td>
<td>1125.73 ± 186.24</td>
<td>1113.64 ± 216.15</td>
<td>1069.27 ± 137.61</td>
<td>0.39</td>
<td>-56.45 ± 151.41</td>
<td>-3.73 ± 13.47</td>
</tr>
</tbody>
</table>

All p-values for effect of time point in one-way repeated ANOVA. All F(2,20). All additional data are reported as mean ±SD. ANOVA, analysis of variance; HDL-C, HDL cholesterol; LDL-2b-P, LDL-2b particles; LDL-3-P, LDL-3 particles; LDL-4-P, LDL-4 particles; LDL-C, LDL cholesterol; Non-HDL-C, non-HDL cholesterol; Non-HDL-P, non-HDL particles; RLP-P, remnant lipoprotein particles; TC, total cholesterol; Total HDL-P, total HDL particles; Total LDL-P, total LDL particles; VLDL-P, very-low-density lipoprotein particles.
P, total LDL-P, LDL-3-P, LDL-4-P, total HDL-P, HDL2b-P, and non-HDL-P did not differ significantly over the 8-week period. Remnant lipoprotein particles (RLP-P) decreased throughout the study, with an overall decrease of 15.5% ($p = 0.03$ for repeated ANOVA; Fig. 2). Results of post-hoc comparisons between time points confirm that only the baseline-to-week 8 change in RLP-P is significant ($p = 0.02$). Inclusion of baseline covariates had no appreciable effects on any of the outcome measures (data not presented).

Exercise frequency, body mass index, and lifestyle factors

None of the participants reported a change in exercise frequency by more than one level. Mean BMI was 28.02 ± 5.36 kg/m² at baseline and 27.76 ± 5.65 kg/m² at the study end point, a decrease of 0.91%. Exercise level and BMI were not found to have any significant impact on study outcomes. Although participants were excluded if they were planning on initiating dietary or lifestyle changes during the study period, one participant notified the study staff at his week 8 (study end) visit that he had gone on vacation for several days and had consumed more alcohol than usual in the days preceding his final visit. This individual had outlying levels of hs-CRP as noted previously.

Discussion

This study aimed to assess the effect of the probiotic yeast *S. boulardii* on cardiovascular biomarkers, including lipids and lipoprotein particles, for the first time in human volunteers. After 8 weeks of daily supplementation, it was found that *S. boulardii* significantly reduced remnant lipoprotein particles, triglyceride-rich lipoproteins, which are closely related to very low-density lipoprotein. Remnant lipoproteins, like LDL, are considered highly atherogenic and are positively correlated with the severity and progression of CAD, independent of LDL-C levels. Levels of remnant lipoprotein have been shown to be positively correlated with impaired endothelial function in human coronary arteries and to be significant predictors of cardiovascular events. Because of their independent atherogenicity, remnant lipoproteins were previously identified by the National Cholesterol Education Program—Adult Treatment Panel III as a strong candidate for interventions aimed at reducing CAD risk.

*S. boulardii* may have the ability to alter cholesterol levels through a mechanism involving cholesterol assimilation. *S. boulardii* has been shown to remove cholesterol from laboratory culture medium by assimilation into the yeast cells. Therefore, it may be possible for *S. boulardii* to assimilate intestinal cholesterol and subsequently alter serum cholesterol levels.

This study was designed to collect preliminary evidence on the efficacy of a specific strain of *S. boulardii* (*Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079) as a treatment for hypercholesterolemia, but eight weeks of daily supplementation with *S. boulardii* was not associated with alterations in total, LDL, and HDL cholesterol. Previous studies that examined the potential hypocholesterolemic effect of other probiotics have yielded mixed results and this may be a reflection of the broad range of probiotic species and strains, dosages, and intervention lengths studied.

Participants in this study had good compliance, as measured by the end-of-study count of returned capsules. They

![FIG. 1. Mean levels of total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triglycerides (TG) at baseline, after 4 weeks, and after 8 weeks.](image)

![FIG. 2. Mean level of remnant lipoprotein particles (RLP-P) at baseline, after 4 weeks, and after 8 weeks. $p$-Value is for a one-way repeated measures ANOVA, with three measurements. Significant difference between time points noted by *$p<0.05$. ANOVA, analysis of variance.](image)
reported very little change in exercise frequency during the study and their BMIs were consistent between baseline and the study endpoint. The results of the study demonstrate the feasibility of many of the study methods; however, limitations included not observing the dietary habits of the participants or including a control group. A follow-up study should utilize validated dietary and physical activity assessment tools and include a control group for comparison. The limited sample size in the current investigation may have reduced the ability to capture potentially significant findings; therefore, future investigations should include an a priori sample size calculation. Additionally, the study population consisted of predominantly Caucasian males with hypercholesterolemia, but who were nonsmoking, normotensive, and without diabetes or established cardiovascular disease. Expanding the recruitment criteria to include participants with additional CAD risk factors in a future study may make the results more generalizable to the population at risk for CAD because of the multifactorial nature of the disease.

Conclusions

The effect of S. boulardii on lipids and lipoprotein particles had not previously been investigated in human volunteers, and the most promising result from this investigation was the finding that S. boulardii supplementation lowered remnant lipoprotein, a predictive biomarker and potential therapeutic target in the treatment and prevention of CAD. Further investigation is needed to confirm this finding, expound upon other potential alterations in cholesterol subspecies, and explore the mechanisms involved.

Acknowledgments

This work has been supported by the Helfgott Research Institute at the National College of Natural Medicine and the National Institutes of Health National Center for Complementary and Alternative Medicine (5R25AT002878). We would like to thank Pure Encapsulations, Inc., and SpectrCell Laboratories for donating the probiotic capsules and their laboratory services, respectively. We also thank the study participants for their time and dedication.

Author Disclosure Statement

No competing financial interests exist.

References


Address correspondence to:
Jennifer Joan Ryan, ND, MS
Helfgott Research Institute
National College of Natural Medicine
2220 SW 1st Avenue
Portland, OR 97201
E-mail: jryan@ncnm.edu

Phase II trial of encapsulated ginger as a treatment for chemotherapy-induced nausea and vomiting. Support Care Cancer. 2009.

Phase II study of the effects of ginger root extract on eicosanoids in colon mucosa in people at normal risk for colorectal cancer.
Phase II Study of the Effects of Ginger Root Extract on Eicosanoids in Colon Mucosa in People at Normal Risk for Colorectal Cancer

Suzanna M. Zick1, D. Kim Turgeon3, Shaiju K. Vareed6, Mack T. Ruffin1, Amie J. Litzinger1, Benjamin D. Wright1, Sara Alrawi1, Daniel P. Normolle2, Zora Djuric1, and Dean E. Brenner3,4,5

Abstract

Inhibitors of COX indicate that upregulation of inflammatory eicosanoids produced by COX, and in particular prostaglandin E2 (PGE2), are early events in the development of colorectal cancer (CRC). Ginger has shown downregulation of COX in vitro and decreased incidence/multiplicity of adenomas in rats. This study was conducted to determine if 2.0 g/d of ginger could decrease the levels of PGE2, 13-hydroxy-octadecadienoic acids, and 5-, 12-, and 15-hydroxyeicosatetraenoic acid (5-, 12-, and 15-HETE), in the colon mucosa of healthy volunteers. To investigate this aim, we randomized 30 subjects to 2.0 g/d ginger or placebo for 28 days. Flexible sigmoidoscopy at baseline and day 28 was used to obtain colon biopsies. A liquid chromatography mass spectrometry method was used to determine eicosanoid levels in the biopsies, and levels were expressed per protein or per free arachidonic acid. There were no significant differences in mean percent change between baseline and day 28 for any of the eicosanoids, when normalized to protein. There was a significant decrease in mean percent change in PGE2 (P = 0.05) and 5-HETE (P = 0.04), and a trend toward significant decreases in 12-HETE (P = 0.09) and 15-HETE (P = 0.06) normalized to free arachidonic acid. There was no difference between the groups in terms of total adverse events (P = 0.55).

On the basis of these results, it seems that ginger has the potential to decrease eicosanoid levels, perhaps by inhibiting their synthesis from arachidonic acid. Ginger also seemed to be tolerable and safe. Further investigation in people at high risk for CRC seems warranted.

Cancer Prev Res; 4(11); 1–9.

©2011 AACR.

Introduction

Anti-inflammatory agents such as aspirin and related nonsteroidal anti-inflammatory drugs (NSAID), which inhibit COX enzymes and decrease the levels of the inflammatory prostaglandin E2 (PGE2) seem to be promising colorectal cancer (CRC) chemopreventive agents (1). Aspirin and related NSAIDs have been shown to prevent the development of adenomas and CRC in both animal models of CRC and in numerous epidemiologic studies (1). Although aspirin and related NSAIDs are encouraging chemopreventive agents, there is some speculation that the inhibition of COX enzymes could cause the shunting of arachidonic acid (AA), the substrate for COX, toward the production of other inflammatory eicosanoids (2).

The lipoygenase (LOX) enzymes also use AA as a substrate to produce eicosanoids. Eicosanoids products of 5-, 12-, and 15-lipoxygenase (5-, 12-, and 15-LOX) are 5-, 12-, and 15-hydroxyeicosatetraenoic acid (HETE; ref. 3). There is some evidence that the simultaneous inhibition of COX-2 and 5-LOX causes greater inhibition of tumor growth and decreased concentrations of PGE2 compared with inhibition of COX-2 or 5-LOX alone (4). While evidence is strongest for the role of PGE2 in colon tumor initiation and progression 5-HETE and 12-HETE have also been implicated in the development of CRC (5). Soumaoro and colleagues showed that 5-LOX expression and 5-HETE concentrations are upregulated in human colorectal cancer specimens, and are correlated with tumor size, depth, and vessel invasion (6, 7). 12-HETE was found to stimulate the proliferation of both HT-29 and HCT-15 human colon carcinoma cells (8). Increased production of 5-HETE and 12-HETE has also been reported in the mucosa of colon cancer patients (9).

Complicating this picture is the production of putative antitumor/anti-inflammatory eicosanoids in colorectal tumorigenesis such as 15-HETE and 13-hydroxy-octadecadienoic (13-HODE) acids (10, 11). Although both 15-HETE
and 13-HODE are catalyzed from different substrates (AA for 15-HETE and linoleic acid for 13-HODE) by 15-LOX, they are produced by different isoforms of 15-LOX, with 15-LOX-1 metabolizing linoleic acid and 15-LOX-2 metabolizing AA (see Fig. 1) (3, 10). Consequently, an attractive colorectal chemopreventive agent would impact not just the COX enzymes, but the balance of products from differing eicosanoid enzymes, potentially shifting the eicosanoid system toward a local anti-inflammatory state.

Ginger root (*Zingiber officinale* Roscoe, Zingiberaceae) is one of the most heavily consumed dietary substances in the world and is one of the top selling dietary supplements in the United States (12, 13). Ginger’s mechanism of anticarcinogenesis action is not entirely known, but seems to be associated with the antioxidant and anti-inflammatory actions of its nonvolatile pungent components the gingerols and shogaols (12). Ginger inhibits 5-LOX (14–17) and COX-1 and -2; (14, 18–20) decreases inflammation in various murine models (12, 16, 21–23), and reduces serum concentrations of PGE$_2$ in rats (23). Several studies of chemically induced colon carcinogenesis have shown that ginger is preventive (24–26). When ginger was administered in the postinitiation stage, however, it did not suppress aberrant crypt foci formation nor did ginger significantly change the proliferative or apoptotic indexes of the colonic crypt (27).

The purpose of this study was to examine the effect of 2.0 g of ginger taken daily for 28 days compared with placebo on eicosanoids in the colon mucosa of people at normal risk for developing colorectal cancer. Secondary objectives were to evaluate the safety, tolerability, adherence, and blinding success of ginger given orally for 28 days.

**Methods**

**Participants**

A total of 33 participants were recruited from the surrounding community through fliers or word-of-mouth between April 2007 and May 2008. To be eligible for the

![Figure 1. Metabolism of linoleic and arachidonic acid.](image-url)
study, participants had to be 18 years or older and in good health as defined by an unremarkable medical history, physical, and screening blood work (chemistry screen, complete blood count) within 60 days of study entry. No chronic medication use was allowed and participants could not have taken aspirin or related NSAIDs during the study or 14 days before the first dose of the study medication. Participants also had to be classified as being at normal-risk for developing colorectal cancer. Normal-risk was defined as having: no history of familial colorectal cancer syndromes; no first-degree relatives with colon cancer diagnosed before the age of 60; no personal history of colorectal cancer; no history of familial colorectal cancer risk for developing colorectal cancer. Normal-risk was participants also had to be classified as being at normal-risk for developing colorectal cancer.

14 days before the first dose of the study medication. Participants were asked to avoid all foods containing ginger within the 14 days prior to drug administration. This was confirmed by having participants complete a food checklist to verify that they were not consuming any ginger-rich foods such as ginger ale or Japanese food. All of the participants were reimbursed for their time. All study procedures were administered at the University of Michigan Clinical Research Unit (MCRU) after the participant gave written, informed consent. The study was approved by the University of Michigan Institutional Review Board.

Drug

The ginger product used in this study was manufactured by Pure Encapsulations. Pure Encapsulation's ginger (Z. officinale) powder was processed using Good Manufacturing Procedures (GMP). Each capsule contained 250 mg dry extract of ginger root [10:1 (v/v) extraction solvent (ethanol 50%): root], normalized to 15 mg (5%) of total gingerols. On the basis of high performance liquid chromatography (HPLC) analysis, a 250 mg capsule of ginger extract (from both batches) contained 5.38 mg (2.15%) 6-gingerol, 1.80 mg (0.72%) 8-gingerol, 4.19 mg (1.78%) 10-gingerol, and 0.92 mg (0.37%) 6-shogaol. Gingerol and shogaol content was verified by an independent laboratory using appropriate HPLC techniques (Integrated Biomolecule Corporation). The study was conducted using 2 batches (ZO/06006 and ZO/07006) of ginger powder extract, both of which were tested for gingerols and shogaol content.

The 2.0 g dose used in the study was chosen based on the highest tolerated amount of ginger extract in a phase I dose escalation study in healthy volunteers (28). Also, 2.0 g of ginger extract is equivalent to 20 g of raw ginger root, which would be a large but not unreasonable amount to consume through the diet. Placebo consisted of lactose powder. Ginger powder and lactose were placed into identical opaque red capsules. Placebo and ginger capsules were assembled, stored, and dispensed by the Investigational Drug Service of the University of Michigan (U of M IDS). The participants were instructed to take eight 250 mg capsules daily with food and to bring any unused capsules to the final (28 day) study visit.

Randomization, allocation, and blinding

Eligible participants were randomized equally to one of 2 groups: placebo or ginger extract (2.0 g). The randomization code was computer generated by the study bio-statistician. The randomization code was kept by the University of Michigan (U of M IDS), which assigned the next available randomization number as the study team informed them of eligible participants. Study participants and all study personnel who assessed outcomes, worked with study data or administered tests or questionnaires were unaware of the randomization list or treatment assignment.

Adherence and assessment of blinding

Participants were assessed for adherence by a research coordinator through weekly telephone calls, self-report, and pill counts at the end of the study. Adherence was defined as taking the capsules within 4 hours of the agreed upon time, twice daily. Participants were classified as adherent if the adherence monitoring suggested that 80% or more of the doses were taken as prescribed.

Blinding was assessed by asking the participants during their final visit which treatment they believed they received ("ginger," "placebo," or "do not know"). Participants were also asked the reason for their response, for example, "Was it the way the capsule smelled?"

Toxicity assessment

Participants were assessed for toxicity by direct questioning in person, by email or by telephone at weekly intervals. The National Cancer Institute (NCI) Common Toxicity Scale V 3.0 (Regulatory Affairs Branch, Cancer Therapy Evaluation program, Division of Cancer Treatment, Diagnosis, and Centers, NCI; ref. 29) was used to quantify toxicity.

Flexible sigmoidoscopy and tissue collection

Participants underwent 2 flexible sigmoidoscopies, one before drug treatment and the second 28 days after ginger extract treatment commenced. The second procedure was done at a time as close as possible to 24 hours after the participant took the final ginger dose. The participants were not prepared for the procedure with any enemas. Participants were, however, asked to evacuate their rectum within 12 hours of the procedure, but to not take any laxatives to enhance evacuation.

Participants were placed in a left lateral decubitus position and a flexible sigmoidoscope was passed to 20 to 25 cm from the anal sphincter. Four tissue samples were taken by opening and pressing the biopsy forceps perpendicular to the mucosal surface with mild pressure. Each biopsy specimen was taken approximately 2 cm or more from other biopsy sites in distal sigmoid colonic mucosa that had no visual appearance of trauma or recent biopsy.
Tissue handling and disposition

Biopsy samples were placed into a sterile 1.5-mL Eppendorf tube and frozen in liquid nitrogen at exactly 50 seconds after the time the biopsy forceps were closed. The specimens were stored at −70°C until immediately before analysis.

Frozen biopsy samples weighed approximately 5 mg and yielded between 400 and 600 µg protein. Triplicate assays for the eicosanoids required approximately 10 to 20 µg of colon tissue. The remaining frozen tissue samples were stored at −70°C for future use.

Analytical methods eicosanoids (PGE₂, 5-HETE, 12-HETE, 15-HETE, and 13-HODE)

All eicosanoids and deuterated internal standards used in this study were purchased from Cayman Chemical Co. Arachidonate, butylated hydroxytoluene (BHT), citric acid, and EDTA were obtained from Sigma Chemical Co. All Burdick and Jackson brand HPLC-grade solvents were purchased from Fisher Scientific Co.

Reverse-phase LC electrospray ionization mass spectrometry (LC/MS/MS) analyses were used for quantitation of PGE₂, 5-HETE, 12-HETE, 15-HETE, and 13-HODE as described previously (30, 31). Two frozen colonic biopsy specimens from the same participant and time point were removed from the freezer, combined and ground to a fine powder using a liquid-nitrogen–cooled mortar. Samples were then transferred to sealed microcentrifuge tubes, and 3 volumes of ice-cold PBS buffer containing 0.1% BHT and 1 mmol/L EDTA were added. The samples were then homogenized by an Ultrasonic Processor (Misonix) at 0°C for 3 minutes. A 100-µL aliquot of the homogenate was transferred to a glass tube (13 x 100 mm) for extraction of eicosanoids. Briefly, 20-µL aliquots of 1 N citric acid and 10 µL of deuterated PGE₂, 5-, 12-, or 15- HETE; or 13-HODE (100 ng/mL) were added to the samples. Eicosanoids were then extracted with 1 mL of hexane:ethyl acetate (1:1, v/v) and vortexed for 2 minutes. All extraction procedures were done at minimum light levels under cold conditions (4°C). Samples were centrifuged at 1800 × g for 10 minutes at 4°C. The upper organic layer was collected, and the organic phase from 3 extractions were combined before drying under a stream of nitrogen at room temperature. Samples were then reconstituted in 100 µL of methanol:ammonium acetate buffer (10 mmol/L at pH 8.5; 70:30, v/v) before LC/MS/MS analysis. The protein concentration in the homogenate was determined by a Bradford protein assay (Bio-Rad).

LC/MS/MS analyses were done using a Quattro Ultima tandem mass spectrometer (Micromass) equipped with an Agilent HP 1100 binary pump HPLC inlet. Eicosanoids were separated using a Luna 3 μ Phenyl-Hexyl 2 × 150 mm LC column (Phenomenex). The mobile phase consisted of 10 mmol/L ammonium acetate (pH 8.5) and methanol. For the analysis of PGE₂, HETEs, and 13-HODE, the separation was achieved using a linear methanol gradient from 40% to 60% more than 18 minutes followed by a methanol flush. The flow rate was 250 µL/min with a column temperature of 50°C. The sample injection volume was 25 µL. Samples were kept at 4°C during the analysis. All eicosanoids were detected using electrospray negative ionization and multiple-reaction monitoring of the transition ions for the metabolites and their internal standards (32).

The mass spectrometer (Thermo Finnigan TSQ Quantum) was operated in the electrospray negative ion mode with a cone voltage of 2,300 V, a cone gas flow rate of 117 L/h, and a deviation gas flow rate of 998 L/h. The temperature of the desolvation region was 350°C, and the temperature of the source region was 120°C. Fragmentation for all compounds was done using argon as the collision gas at a collision cell pressure of 2.10 × 10⁻³ Torr. The collision energy ranged from 16 to 31 V depending on the analyte. The results were either expressed as nanogram (ng) of eicosanoid per milligram (mg) of protein or as ng of eicosanoid per microgram (µg) of free AA. All of the biopsy samples from a given individual were assayed in the same batch to eliminate any batch effects on changes over time. Four batches were assayed for this study. The within-day coefficients of variation (CV) of the assay, based on 3 injections of the same sample on the same day, for PGE₂, 5-, 12-, 15-HETE, 13-HODE, and AA were 3.8%, 13.2%, 15.4%, 13.2%, 12.2%, and 2.5%, respectively and the between-day CV for PGE₂, 5-, 12-, 15-HETE, 13-HODE, and AA were 5.3%, 18.9%, 16.0%, 34.2%, 28.4, and 7.0%, respectively.

Statistical methods and sample size

Statistical analyses were conducted using SPSS software version 18.0. Baseline characteristics were analyzed, stratified by treatment group, using means and SDs for continuous variables, and counts and percentages for categorical variables. Balance between treatment groups on baseline characteristics was tested using independent sample t tests for continuous variables and Pearson’s χ² and Fisher’s exact tests, as appropriate, for categorical variables.

Mean percent change from baseline to day 28 for each treatment group for PGE₂, 5-HETE, 12-HETE, 15-HETE, and 13-HODE was calculated [e.g., (PGE₂ at day 28—PGE₂ at baseline)/PGE₂ at baseline]. Also, given the large batch-to-batch variability of the eicosanoid assays, we investigated the effect of batch on mean percent change using general linear models. A Kolmogorov-Smirnov-Lilliefors’ (KSL) test for normality was conducted to determine if either treatment groups were normally distributed. Depending on the results of the KSL test, independent sample t tests were used to calculate the differences between treatment groups for mean percent change when normally distributed and the Mann–Whitney U test was used when not normally distributed. Results are reported as means ± SD. Adverse events (AE), blinding, and adherence between groups were analyzed using Pearson’s χ² or Fisher’s exact test as appropriate. A P ≤ 0.05 was considered statistically significant.

The sample size needed for the study was determined using published data on PGE₂ levels (33). PGE₂ concentration in human colon tissue at baseline had a mean and SD of 11.7 pg/mg. ± 1.7 pg/mg. On the basis of this PGE₂
level, we calculated that a sample size of 15/treatment group would have better than 80% power to detect a reduction in PGE\textsubscript{2} level of at least 25%. A post-hoc power analysis was also done for PGE\textsubscript{2}. The analysis was based on the observed data using a 2-sample \textit{t} test of percent change for PGE\textsubscript{2} to determine the sample size needed for 80% power.

**Results**

**Subjects and toxicity**

We screened 50 people between January 2007 to June 2008, of whom 33 met all eligibility criteria and were randomized: 17 to placebo and 16 to 2.0 g ginger, for 28 days. Figure 2 documents the numbers of participants, reasons for exclusions and reasons for discontinuing the intervention. There was no significant difference between treatment groups for any demographic or clinical characteristic. Less than one-half of the participants were male (n = 16, 48.5%) with mean (±SD) age of 33.9 ± 11.5 (range 20–59 years), and more than one-half (n = 21, 63.6%) of the participants were Caucasian. Less than one-fifth were African American (n = 6, 18.2%) and only 3% (n = 1) of participants reported being of Hispanic ethnicity. The mean body mass index (BMI) was 25.9 ± 5.0 (range 18.2–39.3).

All toxicities reported are shown in Table 1. No toxicities greater than NCI Common Toxicity Criteria (v. 3.0) Grade 1 was reported (29). There was no difference between the groups in terms of total AEs (\(P = 0.55\)) or specific types of AEs such as gastrointestinal (GI) toxicities (\(P = 0.71\)).

**Eicosanoids (PGE\textsubscript{2}, 5-HETE, 12-HETE, 15-HETE, and 13-HODE)**

The baseline values of PGE\textsubscript{2}, 5-HETE, 12-HETE, 15-HETE, and 13-HODE in colon biopsies across both groups were 10.8 ± 10.3, 0.7 ± 0.5, 0.8 ± 0.6, 7.8 ±...
5.0, and 27.1 ± 19.1 pg/µg protein, respectively (mean ± SD, n = 30). Table 2, presents all continuous outcomes (PGE2, 5-, 12-, 15-HETE, and 13-HODE, AA), and mean percent change from baseline to day 28 of PGE2, 5-, 12-, 15-HETE, 13-HODE, and AA. Table 2 presents eicosanoid concentrations normalized by protein mass, the, the conventional method used to report ELISA results, and normalized by the mass of AA. The LC/MS/MS method also detects and quantifies AA.

The mean percent change in PGE2 in the colon mucosa after 28 days, as compared with placebo, was significantly lower (−28% vs. +26%, P = 0.05), as shown in Table 2. When PGE2 was normalized to protein the results were not significant (P = 0.16), but there was a trend suggesting that ginger decreased PGE2 compared with placebo (7% vs. +32%, Table 2).

Changes in the other eicosanoids also were more evident when normalized to AA levels. There were no significant differences in mean percent change between baseline and day 28 for any of the other eicosanoids (HETE-5, -12, -15, and 13-HODE), when normalized to protein. In contrast, there was significant decrease in 5-HETE (P = 0.04) compared with placebo and trends toward significant decreases in 12-HETE (P = 0.09) and 15-HETE (P = 0.06) when eicosanoid concentrations were normalized to AA. There was no significant effect of batch (P = 0.47–0.95) on mean

Table 2. Eicosanoids levels in normal mucosa in participants at normal risk for colorectal cancer [mean (SD)]

<table>
<thead>
<tr>
<th>Eicosanoid</th>
<th>Baseline</th>
<th>28-day follow-up</th>
<th>Mean % change (SD) BTWN baseline and week 4</th>
<th>28-day follow-up</th>
<th>Mean % change (SD) BTWN baseline and week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGE2</td>
<td>9.1 (10.5)</td>
<td>8.6 (6.9)</td>
<td>31.9 (89.8)</td>
<td>12.9 (10.1)</td>
<td>10.8 (10.0)</td>
</tr>
<tr>
<td>5-HETE</td>
<td>0.6 (0.4)</td>
<td>0.9 (1.1)</td>
<td>54.9 (190.5)</td>
<td>0.8 (0.7)</td>
<td>0.9 (0.9)</td>
</tr>
<tr>
<td>12-HETE</td>
<td>0.8 (0.7)</td>
<td>1.5 (2.8)</td>
<td>71.5 (158.8)</td>
<td>0.8 (0.6)</td>
<td>1.3 (2.1)</td>
</tr>
<tr>
<td>15-HETE</td>
<td>7.4 (5.0)</td>
<td>12.7 (22.0)</td>
<td>63.3 (171.0)</td>
<td>8.2 (5.1)</td>
<td>11.4 (19.3)</td>
</tr>
<tr>
<td>13-HODE</td>
<td>22.1 (15.4)</td>
<td>22.4 (15.3)</td>
<td>17.7 (75.0)</td>
<td>32.8 (21.8)</td>
<td>27.7 (23.1)</td>
</tr>
<tr>
<td>AA (ng/µg)</td>
<td>0.7 (0.4)</td>
<td>0.8 (0.6)</td>
<td>24.1 (84.7)</td>
<td>0.7 (0.3)</td>
<td>1.3 (1.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGE2</td>
<td>13.1 (11.5)</td>
<td>11.5 (7.6)</td>
<td>26.4 (96.0)</td>
<td>18.2 (12.1)</td>
<td>12.9 (12.2)</td>
</tr>
<tr>
<td>5-HETE</td>
<td>1.0 (1.0)</td>
<td>1.0 (0.6)</td>
<td>21.5 (58.5)</td>
<td>1.2 (0.8)</td>
<td>10.4 (0.7)</td>
</tr>
<tr>
<td>12-HETE</td>
<td>1.2 (1.0)</td>
<td>1.4 (1.0)</td>
<td>41.0 (57.8)</td>
<td>1.2 (0.8)</td>
<td>1.3 (1.1)</td>
</tr>
<tr>
<td>15-HETE</td>
<td>9.3 (5.9)</td>
<td>11.2 (7.9)</td>
<td>26.7 (61.7)</td>
<td>11.0 (6.1)</td>
<td>9.6 (8.2)</td>
</tr>
<tr>
<td>13-HODE</td>
<td>37.1 (34.2)</td>
<td>32.5 (20.8)</td>
<td>9.7 (69.8)</td>
<td>50.6 (34.4)</td>
<td>38.1 (32.3)</td>
</tr>
</tbody>
</table>

aIndependent t test or
bMann–Whitney U test, as appropriate, on the difference between mean percent change between baseline and day 28.

Mean percent change between baseline and week 4 is calculated as [(eicosanoid at time 2/eicosanoid at time 1)/eicosanoid at time 1] per participant and then an average is obtained. Mean percent change may not seem reflective of change in baseline and 28-day follow-up mean values. This is due to the large amount of variability in the baseline measures.

Table 1. Adverse events reported by person

<table>
<thead>
<tr>
<th>AE</th>
<th>Ginger (n = 14)</th>
<th>Placebo (n = 16)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All participants with any AE, no. (%)</td>
<td>5 (35.7)</td>
<td>7 (43.8)</td>
<td>0.55</td>
</tr>
<tr>
<td>GI</td>
<td>5 (35.7)</td>
<td>6 (37.5)</td>
<td>0.71</td>
</tr>
<tr>
<td>Headache</td>
<td>1 (7.14)</td>
<td>1 (6.25)</td>
<td>0.83</td>
</tr>
<tr>
<td>Fatigue</td>
<td>0 (0)</td>
<td>2 (12.5)</td>
<td>0.47</td>
</tr>
<tr>
<td>Other</td>
<td>0 (0)</td>
<td>2 (12.5)</td>
<td>0.47</td>
</tr>
</tbody>
</table>

NOTE: All AEs are NCI grade 1.

P: x² or Fisher’s exact test as appropriate.

GI symptoms include: bloating, gas, nausea, heartburn, mouth burning, rectal itching, stomach pain, IBS.

Other includes: flu symptoms, pain in leg.
percent change for any eicosanoid whether normalized to protein or free AA.

**Blinding and adherence**

Participants were able to determine whether or not they had received ginger compared with placebo ($P = 0.02$). Participants who were randomized to placebo were unable to correctly guess their group assignment (44% guessed they were taking ginger). In contrast, participants who received ginger correctly guessed "ginger" 86% of the time. We also asked participants, "Was it the way the capsule worked, tasted, looked or smelled?" that helped you guess what you were taking? Only the way in which the capsule tasted was significantly different ($P = 0.01$) between treatment groups.

All participants were adherent per our definition of taking at least 80% of their capsules. Participants on average took 100 ± 9.9% of their capsules and there was no difference between study groups ($P = 0.15$).

**Discussion**

We found a significant effect of a ginger root extract, in the dose and formulation used, to decrease our primary endpoint, the mean percent change in PGE$_2$ levels in colon biopsies from subjects at normal risk for developing colorectal cancer when normalized to free AA. We did not, however, find a significant difference in PGE$_2$ concentrations when normalized to protein. Similarly, we found no difference in the concentrations of 5-, 12-, 15-HETE, or 13-HODE when normalized per protein. However, when normalized per free AA there was a significance decrease in 5-HETE and decreases in both 12-, and 15-HETE approached significance. Eicosanoid levels per amount of protein reflect absolute concentrations of eicosanoids in the tissue; however, eicosanoid levels per amount of free AA could reflect enzymatic activity of the COX and LOX enzymes. In essence, when the catalytic enzymes, that is, COX are blocked, less substrate is metabolized increasing the amount of AA and decreasing the eicosanoids. This may possibly imply some inhibition of COX-1, LOX-5, -12, and LOX-15-2 enzymes by ginger extract. However, rigorous kinetic experiments assessing COX and LOX enzymatic activity would need to be conducted to confirm this hypothesis. Linoleic acid was not quantified, making interpretation of 13-HODE levels difficult.

This study observed a 28.0% mean decrease in PGE$_2$ normalized to free AA and a roughly 7% decrease when normalized to protein from baseline colon mucosal levels. To date, how much PGE$_2$ concentration needs to be decreased in human colonic mucosa to prevent the occurrence of adenomas is unknown. While aspirin has been shown to both prevent adenomas and decrease colonic mucosal PGE$_2$, no studies have combined these endpoints. Several studies have examined the effect of aspirin on production of PGE$_2$ in human colonic mucosa showing anywhere from no reduction to an 85% decrease in PGE$_2$ in colonic mucosa (33–37). Unlike aspirin, a study examining sulindac, another NSAID, did examine the effect on mucosal prostanooids and polyp occurrence in patients with genotypically affected familial adenomatous polyposis. On average, PGE$_2$ concentrations in rectal biopsies in participants that received sulindac decreased significantly by 19.2% from baseline levels when taken for 48 months at doses of either 75 to 150 mg daily (38). In the sulindac arm, those participants that did not develop an adenoma had a 33.9% mean reduction in baseline PGE$_2$ rectal mucosal concentrations compared with baseline levels, while those who received sulindac and developed a polyp had a slight increase of 2.4% from baseline PGE$_2$ levels (38). In contrast, taking difluoromethyllornithine/sulindac for 3 years resulted in a 70% to 90% reduction in the recurrence of colorectal adenomas but this was not correlated with reductions in mucosal PGE$_2$, although higher baseline levels of PGE$_2$ were associated with higher recurrence rates (39). Our results are more modest than those observed for aspirin, but only slightly lower than those observed for sulindac. Our results are most likely attenuated by lower baseline PGE$_2$ levels because of our healthy normal risk for CRC sample and relatively short study duration of 28 days. Longer-term use in high-risk patients could possibly maximize the effect of ginger.

Previous to this study, ginger and ginger constituents' anti-inflammatory effects on COX and LOX enzymes and their products had only been observed ex vivo (40). The only exception is in one study of rats where decreased serum levels of PGE$_2$ were observed with ginger treatment (22). The present study indicates that oral ginger could have inhibitory effects on colon tissue COX and LOX enzymes in humans.

This study had several limitations. We had a small sample size of only 30 participants and this study was intended as a pilot to determine whether a larger study with ginger extract was warranted. Also, our results had much larger SD for all of the eicosanoids than anticipated, and as such we had inadequate sample size to detect meaningful changes in colon eicosanoid concentrations in several instances, especially when normalized to protein. The sample size of this study was based on the mean and SD of PGE$_2$ concentrations in human colon tissue determined by our group's previous study using ELISA (33). The ELISA assay results indicated SD of around 10% of the mean. In contrast, the LC/MS/MS assay, employed in our study had SD that exceeded 100% of the mean. With this SD, a post-hoc sample size analysis indicated that 61 subjects would be needed to detect a significant difference in PGE$_2$ levels normalized to protein.

Despite the variability of the LC/MS/MS assay it provided several advantages over an ELISA. Mainly, with LC/MS/MS we could measure numerous eicosanoids and free AA simultaneously. The LC/MS/MS method is also more specific for a given analyte than ELISA as it avoids cross-reactivity issues inherent in ELISAs. Importantly, however, we did determine that our mean baseline PGE$_2$, 12-, 15-HETE, and 13-HODE concentrations per protein derived from LC/MS/MS were similar to other studies (11, 32, 36), which used other methods to determine eicosanoid concentrations. Other studies using ELISA and gas chromatography-mass spectrometry also found high amounts of...
variability in colonic PGE2 concentrations, not dissimilar to our results (38, 41). One explanation for the high level of variability in our eicosanoid assays is more than 15% between-day CV (42) for all the eicosanoids except PGE2 and AA. However, assay batch had no significant effect on mean percent change for any eicosanoid when examined in linear models, and was thus not added to the final analysis. Another source of variability is the considerable dissimilarity of eicosanoids at different locations of the colon both between and within people (41). To help address this, we combined 2 biopsies from the same participant at the same time point, but it was in the same section of the colon.

Participants reported a high level of adherence in this study with an average intake of 100% of study medication, making it an unlikely source of variability. A recent study has also found that adenoma risk was not significantly associated with genetic variation in PGE2 synthase and prostaglandin dehydrogenase, however, genetic variations in these key enzymes and associations with variation in levels of PGE2 were not examined (43). Similarly, no significant associations were found between age, BMI, percentage of body fat, NSAID drug use, history of adenomas, and family history of colon cancer with either baseline levels of mucosal PGE2 or change of PGE2 through time (41). Another potential source of variability could be due to differences in absorption of key ginger constituents in human tissue. Limited research has been conducted examining the pharmacokinetics of ginger constituents in human blood and tissue. In one study, a dose of 2.0 g of ginger extract led to detectable levels of all 4 of the main ginger constituents (6-, 8-, and 10-gingerols, and 6-shogaols) in human plasma after a single oral dose (28). Some normal colon tissue samples were also determined to have detectable levels of 10-gingerol glucuronide and sulfate within 24 hours of the last dose of ingesting 2.0 g of ginger extract for 28 days. Presence of gingerols in tissue were affected by the length of time form the last dose of ginger extract due to the fast half-lives (between 1 to 3 hours) and clearance of the gingerols and shogaol in humans (44). These findings argue for large sample sizes, careful recording of when ginger was last consumed and the use of colonic biopsies taken at multiple time points to help draw meaningful conclusions that would otherwise be masked by the considerable variability in this marker.

Future studies of ginger root extract should focus on examining the mechanisms of action by which ginger extract is affecting the COX and LOX enzymes involved in the production of both the inflammatory and anti-inflammatoary eicosanoids. In addition, the effect of ginger on microsomal prostaglandin E2 synthase-1 (mPGEs-1) and 15-hydroxyprostaglandin dehydrogenase (15-PGDH) should be considered as the role of both of these enzymes in PGE2 production and degradation are being recognized as increasingly important to governing tissue concentrations of PGE2 (45).

Subsequent studies should also examine the effect of ginger extract in people at high risk for CRC to determine if there is a differential or similar effect between normal and high-risk populations.

In conclusion, ginger seemed to be well tolerated. There were no differences between placebo and ginger for total AEs or in common AE categories including fatigue, gastrointestinal effects, or headaches. Participants reported a high level of adherence with all participants reporting taking at least 80% of their study medication. Ginger extract had no significant effect on colon concentrations of AA, PGE2, 5-, 12-, and 15-HETE or 13-HODE normalized to protein when compared with the placebo group. However, ginger extract did seem to have an inhibitory effect on COX and LOX-5, 12-, and 15-2 enzymes as observed by significant or close to significant decreases in the mean percent change in PGE2, 5-, 12-, and 15-HETE normalized to AA. Consequently, it would seem that ginger extract has an anti-inflammatory effect in the colon of persons at normal risk for CRC and warrants further study.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Ananda Sen, PhD, for assistance with statistical analyses and Kate Brummett for assistance with figures.

Grant Support

This publication was made possible in part by Grant Number P30 CA047904, P30 CA 48592, and K07CA102592 from the NCI and University of Michigan Clinical Research Center II.1RR024986, and the Kutsche Family Memorial Endowment. The ginger extract was generously donated by Pure Encapsulations.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 29, 2011; revised August 1, 2011; accepted August 3, 2011, published OnlineFirst October 11, 2011.

References

Ginger’s Effect on Eicosanoids in the Colon of Volunteers


Phase II trial of encapsulated ginger as a treatment for chemotherapy-induced nausea and vomiting.
Support Care Cancer. 2009.
Phase II trial of encapsulated ginger as a treatment for chemotherapy-induced nausea and vomiting

Suzanna M. Zick · Mack T. Ruffin · Julia Lee · Daniel P. Normolle · Rivka Siden · Sara Alrawi · Dean E. Brenner

Received: 14 June 2008 / Accepted: 15 October 2008 © Springer-Verlag 2008

Abstract

Goals of work Ginger has been used to treat numerous types of nausea and vomiting. Ginger has also been studied for its efficacy for acute chemotherapy-induced nausea and vomiting (CINV). However, its efficacy for delayed CINV in a diverse oncology population is unknown.

Materials and methods We performed a randomized, double-blind, placebo-controlled trial in 162 patients with cancer who were receiving chemotherapy and had experienced CINV during at least one previous round of chemotherapy. All participants were receiving a 5-HT₃ receptor antagonists and/or aprepitant. Participants were randomized to receive either 1.0 g ginger, 2.0 g ginger daily, or matching placebo for 3 days. The primary outcome was change in the prevalence of delayed CINV. Secondary outcomes included acute prevalence of CINV, acute and delayed severity of CINV, and assessment of blinding.

Main results There were no differences between groups in the prevalence of delayed nausea or vomiting, prevalence of acute CINV, or severity of delayed vomiting or acute nausea and vomiting. Participants who took both ginger and aprepitant had more severe acute nausea than participants who took only aprepitant. Participants were able to accurately guess which treatment they had received. Ginger appeared well tolerated, with no difference in all adverse events (AEs) and significantly less fatigue and miscellaneous AEs in the ginger group.

Conclusions Ginger provides no additional benefit for reduction of the prevalence or severity of acute or delayed CINV when given with 5-HT₃ receptor antagonists and/or aprepitant.

Keywords Ginger · Aprepitant · Chemotherapy-induced nausea and vomiting

Introduction

Chemotherapy-induced nausea, retching, and vomiting (CINV) has historically had significant negative impacts on the quality of life (QoL) and daily functioning of patients receiving chemotherapy [5]. CINV that occurs within the first 24 h of chemotherapy treatment is considered acute. Delayed CINV occurs at greater than 24 h post-treatment and can persist for several days. The negative impacts of CINV on QoL persist despite the introduction of newer treatments for nausea and vomiting, such as serotonin (5-HT₃) antagonists for acute CINV and...
Several recent surveys have found that the prevalence of CINV after receiving conventional anti-emetic therapy ranged from 48% to 67% [3, 6, 12, 16]. In one survey conducted in ten community oncology clinics, only 33% of the patients did not have either delayed or acute CINV, and the majority of patients who developed CINV experienced both delayed and acute CINV [6]. In a US national survey, only fatigue was a more prevalent side effect of chemotherapy treatment compared to CINV [16]. In particular, patients consistently reported significantly more delayed nausea and vomiting compared to acute CINV, indicating that delayed CINV continues to be difficult to control despite the introduction of anti-emetic agents targeted at this timeframe [6, 13]. Patients experiencing CINV reported lower satisfaction with their care [12], missed work days [16], and negative effects on QoL [3, 6, 12, 16]. Agents that could safely further decrease the rates of CINV, and especially delayed CINV, are needed.

Ginger root (Zingiber officinale Roscoe, Zingiberaceae) was first cultivated in Asia and has been used as a medicinal herb for at least 2,000 years [27]. In Chinese, Indian, Middle Eastern, and western herbal medicine, ginger is used primarily as a remedy for digestive disorders including dyspepsia, nausea, vomiting, and diarrhea [17, 25].

Ginger root contains approximately 1.5% to 2.0% of a number of pungent compounds [4]. Gingerols are the most abundant pungent compounds in fresh roots, and several gingerols of various chain lengths (n6 to n10) are present, with the most plentiful being 6-gingerol. Shogaols, the dehydrated form of gingerols, are mainly found in the dried and thermally treated roots, with 6-shogaol being the most abundant [20]. Gingerols and shogaols appear to be the compounds that confer most of the medicinal properties to ginger root.

Ginger demonstrates numerous properties that may be beneficial in treating CINV, including reversing the inhibitory effect of cisplatin on gastric emptying in rats [15, 31], as a 5-HT3 receptor antagonist [18, 32, 35], and as an antioxidant [1, 22, 34].

Previous clinical trials have examined the effect of ginger on CINV with mixed results, with one study showing no effect [24], another with mixed results [29], and two others with positive outcomes [28, 33] of ginger compared to placebo or metoclopramide. Apart from their mixed outcomes, it is difficult to assess these studies as they are limited by their small sample sizes, no clear identification or quality control of the ginger product used, unclear or limited patient population, and no examination of appropriate ginger dose. Further, only one study [24] investigated delayed nausea and vomiting. Using a well-defined and broad patient sample, a well-characterized ginger product at several doses, adequate sample size, and investigation into both acute and delayed CINV, we conducted a randomized, placebo-controlled, double-blind clinical trial to determine the efficacy of a ginger extract for the treatment of CINV in adults with a histologically confirmed diagnosis of cancer that were currently being treated with chemotherapy.

Materials and methods

Participants

The study protocol and all procedures were approved by the University of Michigan (UM) Medical School Institutional Review Board and all participating clinical sites review boards. All participants provided written informed consent. The study took place between June 2003 and May 2006. Individuals 18 years and older who had a histologically confirmed diagnosis of cancer currently being treated with chemotherapy (for adjuvant, neoadjuvant, curative, or palliative means) were eligible. Patients must also have received at least one previous chemotherapy treatment with the same chemotherapeutic agent and have experienced nausea or vomiting of any severity as a result of that treatment. Patients were recruited from the UM Health System oncology clinics and from ten other sites that were part of the National Cancer Institute’s (NCI’s) Community Clinic Oncology Program (CCOP). Patients were ineligible if they: (a) were receiving multiple-day chemotherapy; (b) were receiving concurrent radiotherapy that was classified as high or intermediate risk of causing emesis (i.e., total body irradiation, hemi-body, upper abdomen, abdominal–pelvic mantle, cranium, or craniospinal irradiation); (c) were taking therapeutic doses of coumadin (individuals on low-dose coumadin to maintain peripheral or central venous catheters were allowed), aspirin (individuals taking low-dose 81 mg aspirin were allowed), or heparin; (d) had a history of a bleeding disorder(s) and those experiencing clinically significant thrombocytopenia; (e) had an allergy to ginger or had taken ginger in the last week; or (f) were nursing mothers, pregnant women, or planning a pregnancy during the study period. Patients were eligible to participate if they were scheduled to have a single-day chemotherapy regime and to receive a 5-HT3 receptor antagonist antiemetic and/or the antiemetic aprepitant.

All potentially eligible participants were approached by a research assistant or nurse after their visit at various oncology clinics, and interested patients were scheduled for their screening visit. The screening visit was within 28 days of when the study medication was administered and could be replaced with a pre-chemotherapy visit if all study procedures were conducted. Written and verbal informed consent were obtained at the beginning of the screening.
Eligible participants were randomly assigned to receive a ginger extract, manufactured by Pure Encapsulations® (Sudbury, MA, USA), 1.0 g (four capsules ginger and four capsules placebo daily), 2.0 g (eight capsules daily), or a matching placebo (eight capsules daily). These doses were chosen based on the manufacturer’s recommendations and on doses used in previous clinical studies using this extract. Each capsule contained 250 mg dry extract of ginger root [10:1 (v/v) extraction solvent (ethanol 50%)/root] standardized to 15 mg (5%) of total gingerols. The University of Michigan Investigational Drug Service placed 250 mg of the Pure Encapsulations® ginger or lactose powder in size “0” red animal gelatin capsules made by Gallipot®. Based on high-performance liquid chromatography (HPLC) analysis, a 250-mg capsule of ginger extract contained 5.38 mg (2.15%) 6-gingerol, 1.80 mg (0.72%) 8-gingerol, 4.19 mg (1.78%) 10-gingerol, and 0.92 mg (0.37%) 6-shogaol. Content of gingerols and 6-shogaol in the study medication were independently verified using appropriate HPLC methods (Integrated Biomolecule; Tuscon, AZ, USA) [2]. Participants were told to take the study medication twice per day with water and to bring all unused capsules to the final (72 h) study visit. The first study drug dose was taken within 1 h of the completion of chemotherapy. Patients were seen at the study clinic at the time of their chemotherapy treatment and 3 days after the end of their chemotherapy treatment.

Objectives and outcomes

Our primary objective was to compare the effect of a low-dose (1.0 g) and a high-dose (2.0 g) powdered ginger root extract versus placebo for reducing the prevalence and severity of delayed nausea and vomiting using a 2-day patient diary based on a modified “Morrow Assessment of Nausea and Emesis” (MANE; we replicated questions for days 2 and 3, not just the first 24 h after chemotherapy treatment). The MANE is a validated questionnaire used to assess the prevalence and severity of vomiting and nausea in a given time period, i.e. 24 h. Patients, along with being asked whether they experienced nausea or vomiting during or after their chemotherapy treatment, are also asked how long in minutes or hours their nausea lasted and how they would describe their nausea or vomiting “at its worst” using a six-point Likert scale (very mild to intolerable) as well as the time period when the “nausea or vomiting was the worst”, e.g., 4–8 h after treatment [26]. Delayed chemotherapy-induced nausea or vomiting was defined as any nausea or vomiting that occurred greater than 24 h after receiving chemotherapy.

The secondary objectives included: (1) comparing the effect of a low-dose (1.0 g) and a high-dose (2.0 g) powdered ginger root extract versus placebo for reducing the prevalence and severity of acute (within 24 h of receiving chemotherapy) nausea and vomiting (assessed with the MANE); (2) assessing the safety of different doses (low versus high) of oral powdered ginger root; and (3) determining if study participants are blinded to study assignment, as well as determining which variables may unblind participants (taste, smell, and decrease in nausea and emesis) during the 3-day study period.

We assessed safety by querying participants verbally about any hospitalizations or adverse events that occurred during any of the 3 days of the study. We also reviewed hospital records to assess the cause of hospitalizations and gather information about laboratory abnormalities. Toxicities were graded based on National Cancer Institute Common Toxicity Criteria version 3.0 for Adverse Events [8]. All study participants had access to 24-h nursing support at their local institution. Any adverse events reported by the participants to their local health care providers or CCOP research staff were promptly reported to UM study personnel and followed up by a direct query to the CCOP research site for additional information.

Randomization, blinding, and allocation

Eligible participants were randomized equally to one of three groups: placebo, ginger extract 1.0 g, or ginger extract 2.0 g. The randomization code blocked by research site was computer-generated by the study biostatistician. Study participants were also stratified at randomization into one of two strata [strata 1=5-HT3 antagonist; strata 2=aprepitant (NK1 antagonist)]. Participants who received a 5-HT3 antagonist plus aprepitant were placed into the aprepitant strata, while those participants that received only a 5-HT3 antagonist were placed in the 5-HT3 strata. The stratification allowed for an equal distribution of the NK1 antagonist (aprepitant) to be distributed equally between the treatment arms.

All study participants as well as all study personnel who assessed outcomes, worked with study data, or administered tests or questionnaires were unaware of the randomization list or treatment assignment.
Statistical methods and sample size

Baseline characteristics were reported, stratified by treatment group, using means and SDs for continuous variables and counts and percentages for categorical variables. Balance between treatment groups on baseline characteristics was tested using Kruskal–Wallis statistics for continuous variables and Pearson’s chi-square and Fisher exact tests, as appropriate, for categorical variables.

Prevalence of delayed and acute nausea or vomiting was calculated as a binary variable, i.e., “yes” if the patient had nausea or vomiting (of any severity) or “no” if the patient had no nausea or vomiting. If participants vomited and/or retched at least once, it was counted as a “yes” for vomiting for that time period. The prevalence of nausea and vomiting was compared separately by treatment arm using Cochran Mantel–Haenszel tests stratified for aprepitant (“yes” or “no”). Logistic regression was also used to model the effect of treatment while controlling for covariates, including: emetogenicity of chemotherapeutic agent (high, moderate, low); aprepitant (“yes” or “no”); and presence or absence of baseline nausea or vomiting (“yes” or “no”) as appropriate for delayed values and presence or absence of acute nausea or vomiting (“yes” or “no”). Analyses of severity of delayed and acute nausea and vomiting were examined as an ordinal outcome. Severity of nausea and vomiting and/or retching was graded on a six-point scale, where 1 equaled very mild and 6 equaled intolerable. These severity analyses were only performed on participants who had experienced nausea or vomiting. Analyses of severity were performed using Cochran Mantel–Haenszel tests between treatment groups (placebo, ginger=1.0 g and ginger=2.0 g) and stratified by apreptant (yes/no). Analyses were conducted according to the intention-to-treat principle; however, no imputation was performed for missing values at day 1, 2, or 3. Data were entered into a central database located at Dartmouth College (Hanover, NH, USA). For all analyses, two-sided tests and a significance level of 0.05 were used. The experiment-wise type I error rate was protected only for the principal outcome measure. No adjustments were made for multiple-hypothesis testing, as the secondary outcomes were viewed as hypothesis-generating.

The sample size was justified in terms of the analysis of between-treatment differences in delayed nausea. The prevalence of delayed nausea in patients taking standard antiemetic therapy was assumed to be 51% for lower dose chemotherapy and 74% for higher dose chemotherapy. If the highest ginger dose caused a 30% relative reduction from placebo in the prevalence of delayed nausea, the study had 75% power to reject the null hypothesis of no treatment effect at a 5% significance level.

Main results

Screening, enrollment, and withdrawals

We screened 4,244 patients, of whom 162 met all eligibility criteria and were randomized: 57 to the placebo, 53 to the 1.0-g ginger dose, and 52 to the 2.0-g ginger dose. Figure 1 documents sources of recruitment for potential participants, reasons for exclusions, and reasons for discontinuing the intervention. The low proportion of recruited patients reflects the broad screening of unselected patients presenting to oncology clinics at all CCOP sites. Forty-six participants in the placebo group, 43 participants in the 1.0-g ginger dose, and 40 participants in the 2.0-g ginger dose arm completed all study visits. Adherence to study medications was moderate to high, with 79% of all participants taking greater than 80% of all study medication and with no significant differences between groups (p=0.80).

Sociodemographic and clinical characteristics

In Table 1, we present the sociodemographic and clinical characteristics of participants by treatment group. There was no significant difference between treatment groups for any demographic or clinical characteristics.

All participants received a 5-HT3 receptor antagonist. Fifty-two participants (32.1%) received aprepitant. Of the 52 participants given aprepitant, 49 (94.2%) were receiving moderate or high emetic risk antineoplastic compared to only three (5.8%) who were being administered low emetic risk antineoplastic agents.

Prevalence and severity of acute and delayed nausea and vomiting

Fifty-eight percent (n=94) of study participants reported experiencing both acute and delayed nausea, while 30.9% (n=50) of participants reported acute vomiting and/or retching and 24.7% (n=40) reported delayed vomiting and/or retching.

There was no significant difference between either of the ginger doses compared to placebo in the prevalence of acute or delayed nausea or vomiting. This observation was consistent when participants were stratified by whether or not aprepitant was prescribed as part of their treatment for CINV (Table 2). Although not significant, participants who received aprepitant and either dose of ginger had more treatment failures compared to those who received apreptant in addition to placebo for both acute and delayed nausea and vomiting.

When not stratified by use of apreptant, there was no significant difference in severity between either the low
dose or the high dose of ginger and placebo, except for delayed nausea. Participants who received the high dose of ginger (2.0 g) reported having significantly more severe episode of delayed nausea compared to both placebo and low-dose ginger (1.0 g; mean±SD: placebo=2.8±1.2, ginger 1.0 g=2.9±1.1, ginger 2.0 g=3.4±1.1; \( p=0.03 \)). Table 3 presents results of the severity of both acute and delayed nausea and vomiting stratified by the use of aprepitant. For those participants who did not receive aprepitant, we observed no significant difference in severity of nausea or vomiting between either dose of ginger or placebo (Table 3). However, participants who were prescribed aprepitant and either dose of ginger had significantly more severe delayed nausea (Table 3).

Participant and assessor blinding

Both patients and research personnel who were responsible for collecting study endpoints from patients (outcome assessors) were asked to evaluate which treatment (1.0 g, 2.0 g, or placebo) the patient received. Outcome assessors were not able to correctly identify which treatment the patient received (\( p=0.27 \)). Patients, however, were significantly (\( p=0.01 \)) more likely to correctly guess the treatment they were given. Patients indicated that it was the “the way the capsule worked” (16\%_{\text{placebo}}, 51\%_{\text{1.0 g}}, 33\%_{\text{2.0 g}}) for each treatment group; \( p=0.12 \) as the most common reason for knowing which treatment they were taking. The taste of the capsule was the second most likely reason given for being able to know what treatment a participant received (9\%_{\text{placebo}}, 25\%_{\text{1.0 g}}, 33\%_{\text{2.0 g}}) for each treatment group; \( p=0.01 \).

Adverse events

We divided adverse events into those that most commonly occurred in the trial, e.g., laboratory abnormalities events, and side effects most often associated with ginger consumption, e.g., GI events. There were no significant differences in total adverse events, non-serious adverse
events, dyspnea, gastrointestinal events, or laboratory abnormalities between treatment groups, although laboratory abnormalities were close to being significantly higher in the placebo and 1.0-g dose (\(p = 0.06\)) compared to the 2.0-g dose. There were significantly more fatigue (\(p = 0.03\)) and miscellaneous adverse events (\(p = 0.02\)) in the placebo group compared to either ginger dose. Nearly all of the adverse events were non-serious and graded as either a 1 or a 2 on the NCI toxicity version 3. Out of the 42 patients who experienced an adverse event, only three were serious: One patient was hospitalized with an upper extremity deep vein thrombosis, another patient was hospitalized after experiencing severe diarrhea and abdominal pain, and another patient hospitalized for anemia, low platelets, and white blood cells. We found no significant difference in serious adverse events between treatment groups (\(p = 0.07\)).

**Discussion**

We found no benefit of a ginger extract, in the doses and formulation used, on our primary end point, the prevalence of delayed nausea and vomiting, when added to contemporary standard antiemetic therapy in cancer patients.
receiving chemotherapy. These results are consistent whether evaluated in unadjusted analyses or in analyses that adjusted for presence or absence of acute nausea, or vomiting, emetogenicity of chemotherapeutic agent, and use of aprepitant. Likewise, ginger extract caused no decrease in the severity of delayed nausea or vomiting. The 2.0-g dose of ginger extract, however, did increase the severity of delayed nausea ($p=0.03$), although when stratifying by use of aprepitant, severity of delayed nausea was only increased when the 2.0-g dose of ginger was taken with aprepitant ($p=0.01$) and not when taken without aprepitant ($p=0.69$).

Similar to delayed CINV, we found that ginger extract did not decrease the prevalence of acute nausea or vomiting

### Table 2 Prevalence of acute and delayed nausea and vomiting

<table>
<thead>
<tr>
<th>Prevalence of treatment failures</th>
<th>Number (%)</th>
<th>$P$ value$^a$</th>
<th>$P$ value$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo group ($n=46$)</td>
<td>Ginger 1.0 g group ($n=43$)</td>
<td>Ginger 2.0 g group ($n=40$)</td>
</tr>
<tr>
<td>Nausea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No aprepitant</td>
<td>23 (50.0)</td>
<td>21 (48.8)</td>
<td>20 (50.0)</td>
</tr>
<tr>
<td>Yes aprepitant</td>
<td>8 (17.4)</td>
<td>12 (27.9)</td>
<td>10 (25.0)</td>
</tr>
<tr>
<td>Delayed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No aprepitant</td>
<td>23 (50.0)</td>
<td>26 (60.5)</td>
<td>17 (42.5)</td>
</tr>
<tr>
<td>Yes aprepitant</td>
<td>8 (17.4)</td>
<td>11 (25.6)</td>
<td>10 (25.0)</td>
</tr>
<tr>
<td>Vomiting</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No aprepitant</td>
<td>11 (23.9)</td>
<td>14 (26.4)</td>
<td>11 (27.5)</td>
</tr>
<tr>
<td>Yes aprepitant</td>
<td>3 (6.5)</td>
<td>5 (11.6)</td>
<td>6 (15.0)</td>
</tr>
<tr>
<td>Delayed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No aprepitant</td>
<td>8 (17.4)</td>
<td>14 (32.6)</td>
<td>7 (17.5)</td>
</tr>
<tr>
<td>Yes aprepitant</td>
<td>1 (2.2)</td>
<td>5 (11.6)</td>
<td>5 (12.5)</td>
</tr>
</tbody>
</table>

$^aP$ values were calculated using Cochran Mantel–Haenszel tests stratified by aprepitant (yes or no)

$^bP$ values are Pearson chi-squares calculated using logistic regression adjusting for emetic risk of the chemotherapeutic agent (high, moderate, low), aprepitant, presence or absence of baseline nausea or vomiting (yes or no) as appropriate for delayed values and presence or absence of acute nausea or vomiting (yes or no) as appropriate for acute measures

### Table 3 Severity of acute and delayed nausea and vomiting

<table>
<thead>
<tr>
<th>Severity$^a$</th>
<th>Mean±SD</th>
<th>Placebo group ($n=46$)</th>
<th>Ginger 1.0 g group ($n=43$)</th>
<th>Ginger 2.0 g group ($n=40$)</th>
<th>P value$^b$ (aprepitant=no)</th>
<th>P value$^b$ (aprepitant=yes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nausea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No aprepitant</td>
<td>2.8±1.3 (n=23)</td>
<td>3.1±1.2 (n=21)</td>
<td>3.0±1.1 (n=20)</td>
<td>0.47</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>Yes aprepitant</td>
<td>3.1±1.5 (n=8)</td>
<td>2.8±1.1 (n=12)</td>
<td>2.8±1.5 (n=10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delayed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No aprepitant</td>
<td>3.0±1.3 (n=23)</td>
<td>3.0±1.1 (n=25)</td>
<td>3.2±1.1 (n=17)</td>
<td>0.69</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Yes aprepitant</td>
<td>2.2±0.7 (n=9)</td>
<td>2.9±1.3 (n=11)</td>
<td>3.9±0.9 (n=9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vomiting</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No aprepitant</td>
<td>3.6±1.4 (n=11)</td>
<td>3.1±1.4 (n=14)</td>
<td>2.9±0.9 (n=11)</td>
<td>0.61</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>Yes aprepitant</td>
<td>4.0±1.7 (n=3)</td>
<td>3.4±0.6 (n=5)</td>
<td>3.7±1.5 (n=6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delayed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No aprepitant</td>
<td>4.0±1.3 (n=7)</td>
<td>2.7±0.9 (n=12)</td>
<td>3.7±1.0 (n=7)</td>
<td>0.88</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td>Yes aprepitant</td>
<td>3.0±0.0 (n=1)</td>
<td>3.0±1.4 (n=5)</td>
<td>3.6±1.3 (n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$Severity of nausea and vomiting graded on a six-point Likert scale graded as 1=very mild, 2=mild, 3=moderate, 4=severe, 5=very severe, and 6=intolerable

$^bP$ values were calculated using Cochran Mantel–Haenszel tests stratified by aprepitant (yes or no)
compared to placebo. Further, ginger extract at both doses did not affect the severity of acute nausea or vomiting. However, when ginger was taken with aprepitant, there was an increase in the prevalence of delayed vomiting, although this did not reach statistical significance \( (p=0.07) \). Otherwise, these results were consistent whether or not a participant had been prescribed aprepitant.

Ginger appeared to be well tolerated. There was no difference between placebo and ginger for all adverse events, for common AE categories including dypsnea and gastrointestinal complaints, or serious adverse events. Despite the lack of statistical significance difference in serious AEs, all of the serious AEs did occur in the low-dose ginger group and approached statistical significance \( (p=0.07) \). There were significantly fewer complaints of fatigue \( (p=0.03) \) and miscellaneous other adverse events \( (p=0.02) \) in the ginger treatment groups versus placebo, and there were borderline significantly fewer laboratory abnormalities in the high-dose ginger arm \( (p=0.06) \). The occurrence of all serious adverse events in the low-dose ginger group should be viewed with caution, but given the small number of serious events (three), this result could be due to chance alone. Similarly, the positive effects of ginger on fatigue could also be attributed to chance due to the small number of events. The wide variety of events in the miscellaneous other and laboratory AE groups appear unlikely to be explicable by a pharmacologic effect of ginger. However, possible pharmacological effects such as ginger root’s anti-inflammatory [14, 19, 20, 23] and antioxidant effects [1, 22, 34] could be responsible for some of the decreased AEs. As a consequence, future studies could be considered to examine the protective effect of ginger on fatigue and laboratory abnormalities experienced during chemotherapy.

Our results are in contrast to three other randomized controlled trials (RCTs) [28, 29, 33] examining the safety and efficacy of ginger root extracts or powder for acute CINV. Two of these studies [28, 29] are only available in abstract form, allowing for no more than limited comparisons with our study. Pace [28] found that ginger significantly decreased an acute nausea symptom score, and Pecoraro et al. [29] determined that participants who received ginger compared to placebo appeared to have a greater complete treatment response of acute CINV, although no statistical analysis is provided for the later study. The results of these studies could differ from ours for numerous reasons, including overestimation of treatment effects owing to the studies’ small sample sizes (41 and 12 participants, respectively), lack of blinding, use of different and non-validated outcome measures to assess the prevalence and severity of CINV, different doses and ginger formulations, and lack of examination of delayed CINV.

The third study that found results in contrast to ours was a crossover RCT comparing ginger to metoclopramide and ondansetron for controlling the incidence of CINV for 24 h after treatment. Ginger powder was found to be as successful as metoclopramide in complete control of CINV, but ondansetron was found to be superior to both of the other two treatments (complete control of nausea was 62% in ginger, 58% in metoclopramide, and 86% with ondansetron) [27]. Unlike our study, the 1.0-g dose of ginger powder was not co-administered with antiemetics, e.g., 5-HT3 receptor antagonists, but instead was given instead of standard antiemetic medications.

In contrast, another RCT crossover study in gynecologic oncology patients receiving cisplatin comparing 1.0 g ginger to placebo and metoclopramide (placebo during the first 24 h and metoclopramide during the next 4 days) found results similar to ours [24].

There was evidence in this study that ginger, when co-administered with aprepitant, increased the severity of delayed nausea. In addition, while not statistically significant, participants prescribed aprepitant and ginger (either dose) had consistently higher prevalence of both acute and delayed nausea and delayed vomiting compared to those participants who received only aprepitant. It is possible that ginger root, similar to other of food and beverages, can alter the rate and extent of drug absorption. Ginger could decrease the absorption of aprepitant by increasing gastric emptying time and intestinal motility and thus decreasing aprepitant’s anti-nausea effects. Ginger extracts and their constituents can shorten food transit time [30], enhance gastrointestinal motility [36], and reverse pyrogallol-induced delay in gastric emptying in rats [15].

Our study had several limitations. First, we were limited by inadequate power to detect small effect sizes for secondary outcomes. Second, we lacked adequate sample sizes to detect differences in the primary and secondary outcomes by treatment with or without aprepitant. Third, we found that participants were able to determine if they were randomized to either of the ginger treatment arms, indicating that how the capsule tasted allowed them to determine which treatment they had received. Three meta-analyses of clinical trials found that when participants are not blinded, there tends to be a moderate overestimate of the effect of the new treatment [7, 9, 11]. Lack of blinding is of particular concern when the outcomes are subjective or “soft”, such as with severity of nausea where ascertainment bias can play a large role. Fourth, we also had a very heterogeneous patient sample. The variability in our patient sample was beneficial, making the results more generalizable to a wide variety of oncology patients. However, our sample heterogeneity also made it more difficult to detect any effects of ginger in any subset of cancer patients or chemotherapy treatment combinations.
In summary, the data from this study indicate that a ginger extract provides no clinical benefit, at the doses evaluated, when given in addition to standard evidence-based contemporary anti-nausea CINV medical therapy to patients receiving chemotherapy. Ginger extract may have a negative interaction when taken with aprepitant on severity of nausea. Ginger may also have positive benefits in decreasing fatigue and non-GI adverse events during chemotherapy.

Acknowledgments This research was supported by NCI grant 1 KO7 CA102592-01, R21AT0001735 from the National Center for Complementary and Alternative Medicine (NCCAM), NCI CN-55124, and NCI U10CA74648 (CCOP Research Base). Research resources were also provided by the General Clinical Research Center of the University of Michigan (M01-RR00042). The ginger extract was generously donated by Pure Encapsulations® (Sudbury, MA).

References

induced nausea and vomiting. ASHP Mid-Year Clinical Meeting, 429E


Pharmacokinetics of 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol and conjugate metabolites in healthy human subjects.
Pharmacokinetics of 6-Gingerol, 8-Gingerol, 10-Gingerol, and 6-Shogaol and Conjugate Metabolites in Healthy Human Subjects

Suzanna M. Zick,1 Zora Djuric,1 Mack T. Ruffin,1 Amie J. Litzinger,1 Daniel P. Normolle,2 Sara Alrawi,1 Meihua Rose Feng,4 and Dean E. Brenner3

Departments of Family Medicine, Radiation Oncology, and Internal Medicine; and College of Pharmacy and Engineering, University of Michigan Medical School, Ann Arbor, Michigan

Abstract

Background: Ginger shows promising anticancer properties. No research has examined the pharmacokinetics of the ginger constituents 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol in humans. We conducted a clinical trial with 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol, examining the pharmacokinetics and tolerability of these analytes and their conjugate metabolites.

Methods: Human volunteers were given ginger at doses from 100 mg to 2.0 g (N = 27), and blood samples were obtained at 15 minutes to 72 hours after a single p.o. dose. The participants were allocated in a dose-escalation manner starting with 100 mg. There was a total of three participants at each dose except for 1.0 g (N = 6) and 2.0 g (N = 9).

Results: No participant had detectable free 6-gingerol, 8-gingerol, 10-gingerol, or 6-shogaol, but 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol glucuronides were detected. The 6-gingerol sulfate conjugate was detected above the 1.0-g dose, but there were no detectable 10-gingerol or 6-shogaol sulfates except for one participant with detectable 8-gingerol sulfate. The Cmax and area under the curve values (mean ± SE) estimated for the 2.0-g dose are 0.85 ± 0.43, 0.23 ± 0.16, 0.53 ± 0.40, and 0.15 ± 0.12 µg/mL, and 65.6 ± 44.4, 18.1 ± 20.3, 50.1 ± 49.3, and 10.9 ± 13.0 µg·h/mL for 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol. The corresponding tmax values are 22.6 minutes, and the analytes had elimination half-lives < 2 hours. The 8-gingerol, 10-gingerol, and 6-shogaol conjugates were present as either glucuronide or sulfate conjugates, not as mixed conjugates, although 6-gingerol and 10-gingerol were an exception.

Conclusion: Six-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol are absorbed after p.o. dosing and can be detected as glucuronide and sulfate conjugates. (Cancer Epidemiol Biomarkers Prev 2008;17(8):1930–6)

Introduction

The ginger root (Zingiber officinale Roscoe, Zingiberaceae) is one of the most heavily consumed dietary substances in the world (1). Ginger was first cultivated in Asia (2) and has been used as a medicinal herb for at least 2,000 years (2). Medicinal references to ginger appear in early Sanskrit and Chinese texts as well as ancient Greek, Roman, and Arabic medical literature (3). In Western herbal medicine, ginger is used primarily as a remedy for digestive disorders, including dyspepsia, colic, nausea, vomiting, gastritis, and diarrhea (4). The dietary prevalence of foods, such as ginger, garlic, soy, curcumin, chilies, and green tea, are thought to contribute to the decreased incidence of colon, gastrointestinal, prostate, breast, and other cancers in Southeast Asian countries (5).

Ginger contains ~1.0% to 3.0% volatile oils and a number of pungent compounds (6). Gingerols are the most abundant pungent compounds in fresh roots, and several gingerols of various chain lengths (n6 to n10) are present in ginger, with the most abundant being 6-gingerol. Shogaols, the dehydrated form of gingerols, are found only in small quantities in the fresh root and are mainly found in the dried and thermally treated roots, with 6-shogaol being the most abundant (7).

Studies in animal models have shown that ginger and its phenolic constituents (i.e., 6-gingerol) suppress carcinogenesis in the skin (1, 8-12), gastrointestinal tract (13), colon (14, 15), and breast (16). Ginger extracts have been tested for both antitumor promotion and apoptotic potential in several in vitro cell lines, including leukemia (17), and gastric (18), prostate (19), ovarian (20), and lung carcinoma (21). The chemopreventive mechanisms of ginger are not well understood but are thought to involve the up-regulation of carcinogen-detoxifying enzymes (22), and antioxidant (23-28) and anti-inflammatory (7, 29-31) activity. Ginger also inhibits nuclear factor-κB activation induced by a variety of agents (10, 32-34) and has been shown to down-regulate nuclear factor-κB-regulated gene products involved in cellular proliferation and angiogenesis, including interleukin 8 (19) and vascular endothelial growth factor (35). These factors have also been shown to promote tumor cell proliferation and angiogenesis and affect apoptotic response in several cancers.
Only a handful of studies in rats have examined the absorption, bioavailability, metabolites, and elimination of ginger constituents (26, 36-39). Only two of the pungent compounds, 6-gingerol and zingerone, have been investigated, and, in two of these studies, 6-gingerol was administered as an i.v. bolus (36, 37), which is unlikely to be reflective of the usual p.o. dosing. No pharmacokinetic studies have been conducted in humans nor have any studies in mammals or in vitro examined the other major pungent constituents, namely 8-gingerol, 10-gingerol, and 6-shogaol.

I.v. bolus studies in rats indicated that the plasma concentration-time curve of 6-gingerol was illustrated by a two-compartment open model (36), and the serum protein binding of 6-gingerol was found to be $>90\%$ (36, 37). In both healthy normal rats and rats with acute renal failure, an i.v. bolus of 6-gingerol was rapidly cleared from the plasma with a terminal half-life ranging from 7.23 minutes to 8.5 minutes (36, 37). The terminal phase of 6-gingerol increased significantly to 11 minutes in rats with acute hepatic failure (37). More than 60$\%$ of a p.o. dose of 50-mg/kg dose of 6-gingerol was excreted as metabolites in the bile (48%) and urine (16%) within 60 hours (38). A 100-mg/kg p.o. dose of zingerone was found to have similar patterns of elimination of 6-gingerol, with 50$\%$ excreted in the feces and 40$\%$ excreted in the urine over 24 hours (26, 39).

When given p.o. in rats, 6-gingerol is readily conjugated in the intestinal epithelium and the liver to (S)-(6)-gingerol-4′-O-β-glucuronide and excreted through the bile (40). Six minor metabolites [vanillic acid, ferulic acid, (S)-(+)-hydroxy-6-oxo-8-(4-hydroxy-3-methoxyphenyl), octanoic acid, 4-(4-hydroxy-3-methoxyphenyl) butanoic acid, and 9-hydroxy[6]-gingerol] have also been detected in the urine (38).

Ginger and its constituents at doses up to 2.0 g daily have shown very low levels of toxicity and high levels of tolerability in both animals and humans, with only mild gastrointestinal complaints being reported (6). However, it is unclear if low levels of toxicity are due to poor p.o. bioavailability or a high degree of safety of pungent ginger constituents (i.e., gingerols and shogaols) in humans. This clinical trial evaluated the pharmacokinetic profile of 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol and their conjugate metabolites at 6 dose levels, 100, 250, 500, 1,000, and 2,000 mg, administered p.o. to 27 healthy human volunteers. The purpose of this study was to: (a) determine if ginger extract standardized to 5$\%$ gingerols, in capsule formulation, is absorbed and biotransformed in humans; (b) assess the human pharmacokinetics of 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol and their conjugate metabolites; and (c) evaluate the safety and tolerability of up to 2.0 g of a single p.o. dose of ginger extract standardized to 5$\%$ gingerols.

Materials and Methods

The ginger product used in this study was manufactured by Pure Encapsulations®. The Pure Encapsulation® ginger (Z. officinale) powder is processed according to Good Manufacturing Practice guidelines. Each capsule contained 250 mg dry extract of ginger root [10:1 (volume for volume) extraction solvent (50% ethanol) to root ratio] standardized to 15 mg (5$\%$) of total gingerols. Based on high-performance liquid chromatography (HPLC) analysis, a 250-mg capsule of ginger extract contained 5.38 mg (2.15$\%$) 6-gingerol, 1.80 mg (0.72$\%$) 8-gingerol, 4.19 mg (1.78$\%$) 10-gingerol, and 0.92 mg (0.37$\%$) 6-shogaol. The gingerol content was verified by an independent laboratory with the use of the appropriate HPLC techniques (Integrated Biomolecule Corp.). The entire study was conducted with a single batch of ginger-powder extract to optimize product consistency. β-17-estradiol acetate and the enzymes β-glucuronidase (Type IX-A from Escherichia coli) and sulfatase (Type H-1 from Helix pomatia) were purchased from Sigma-Aldrich Inc. Sodium phosphate and sodium acetate (American Chemical Society certified) were purchased from Fisher Scientific. Six-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol were purchased from Chromadex. The standards were found to be $>95\%$ pure per HPLC analysis. Pelargonic acid vanillylamide, the internal standard, was obtained from Sigma and is $>97\%$ pure. Acetonitrile, methanol, hexane, and de-ionized water were all HPLC grade (Burdick & Jackson). HPLC-grade ethyl acetate and ammonium acetate were purchased from Fisher Scientific. HPLC-grade acetic acid was obtained from J.T. Baker.

Clinical Trial Design. Twenty-seven healthy volunteers were solicited by advertisement or word of mouth. The participants needed to be 18 y of age or older, in good health, and not taking any chronic medications. The participants were asked to avoid all foods containing ginger within the 14 d prior to drug administration and completed a food checklist to verify that they were not consuming any ginger-rich foods, such as ginger ale or Japanese food. This was a dose-escalation study and, as such, three participants were assigned per dose level, starting at the lowest dose of 100 mg and to each subsequent dose (250 mg, 500 mg, 1.0 g, 1.5 g, and 2.0 g) except for the 1.0-g (N = 6) and the 2.0-g (N = 9) doses. Six additional participants were assigned to the highest tolerated dose, 2.0 g, to ascertain toxicity, and three participants were added to the 1.0-g dose to act as training samples. After the administration of a single p.o. dose, blood was drawn from the participants at baseline and at 15, 30, and 45 min as well as at 1, 2, 4, 6, 10, 24, 48, and 72 hr after ingestion of the ginger. The plasma fraction was separated from the blood immediately and kept at -20°C until assayed. Toxicities were graded based on the National Cancer Institute Common Toxicity Criteria version 2.0 and monitored continuously for the first 10 hr and then 24, 48, and 72 hr after ginger administration. All the participants received meals standardized to fiber, calorie, and fat content throughout the first 24 hr of the study. All study procedures were administered at the University of Michigan General Clinical Research Center after the participant gave written informed consent. The study was approved by the University of Michigan Institutional Review Board.

Extraction of 6-Gingerol, 8-Gingerol, 10-Gingerol, and 6-Shogaol from Plasma. Plasma samples (490 μL) were spiked with 10 μL of various concentrations of combined working standards and 10 μL of the internal standard, pelargonic acid vanillylamide (100 ng/μL).
The samples were diluted with water and extracted with 2.0 mL ethyl acetate/hexane (1:1, volume for volume). After centrifugation, the upper organic layer was removed into a glass vial and dried under a stream of argon. The samples were resuspended in 60 µL of acetonitrile and 40 µL of water. The samples were filtered and then placed into autosampler vials for HPLC quantification.

Enzymatic Hydrolysis of 6-Gingerol, 8-Gingerol, 10-Gingerol, and 6-Shogaol Conjugates. The samples were also assayed for conjugates after incubating the plasma samples with the enzymes β-glucuronidase and sulfatase through the method of Asai et al. (41). For these assays, the plasma samples (500 µL) were mixed with water (500 µL) and the internal standard (10 µL, 100 µg/mL). The samples were then mixed with 50 µL of β-glucuronidase (50 µL, 446 units) in sodium-phosphate buffer (0.1 mol/L, pH 6.8) and 45 µL of sulfatase (45 µL, 51.5 units) in sodium-acetate buffer (0.1 mol/L, pH 5.0), and incubated at 37°C for 1 hr. The samples were then extracted through the extraction procedure given above. To determine the amount of glucuronide and sulfate conjugates in plasma, the samples were incubated separately with the β-glucuronidase and sulfatase enzymes prior to extraction.

Quantitation of 6-Gingerol, 8-Gingerol, 10-Gingerol, and 6-Shogaol in Plasma. Reverse-phase HPLC was used to quantify 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol in plasma. Chromatographic separation was accomplished with the use of a Phenomenex Luna 4.6 × 250 mm, S-5 µm, C18 column that was coupled with a Phenomenex 4.0 × 20 mm, 5 µ, C18 guard column. The mobile phase consisted of 2% ammonium acetate at pH 4.5/59% acetonitrile/39% water (v/v/v); A) and 100% acetonitrile containing 20 mL of 1.0 mol/L ammonium acetate at pH 4.5 (98/2, volume for volume; B). The extracted sample was eluted on a gradient mobile phase starting from 100% A at zero time to 100% B in 15 min in a Waters #4 curve (concave) gradient and then to 100% A in 1 min. This was followed by 100% reagent B for 5 min and completed with a column wash of 100% reagent A for 10 min at a flow rate of 0.8 mL/min. The injection volume was 20 µL, and detection was done with electrochemical detection at 600, 550, and 500 mV; UV detection was done at 282 nm.

Standard curves were constructed with the use of plasma spiked with 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol. Plasma samples with no detectable ginger analytes were spiked with varying amounts of a standard solution of the four analytes (0.10, 0.25, 0.5, 1.0, 2.5, and 5.0 µg/mL). Each sample was analyzed in duplicate.

Analytical Assessment/Quality Control. Six-gingerol, 8-gingerol, 10-gingerol, 6-shogaol, and the internal standard (p-araconic acid vanillylamide) were well resolved by HPLC. A linear relationship between 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol plasma concentration and response was found in the concentration ranges 0.1 µg/mL to 5.0 µg/mL. The intraday accuracy ranged from 91% to 128%, and the precision was ±11.7% for all four analytes; the interday accuracy ranged from 91% to 113%, and the precision was ±11.7% at three concentrations (5.0, 1.0, and 0.25 µg/mL) for all four analytes. The interday coefficients of determination (R²) spanned from 0.9894 to 0.9992 for all four analytes and were observed over 4 d with gingerol-spiked and shogaol-spiked plasma. The lower limit of quantitation for this method was 0.1 µg/mL, except for 10-gingerol, which was 0.25 µg/mL. The extraction efficiency of all four analytes at 5.0, 1.0, and 0.25 µg/mL concentration levels derived from area ratios ranged from 82.5% to 165.3%, with extraction efficiencies >100%. This may be due to variability in the chromatographic peak quantitation and in the standard curve variances, leading to experimental error. The intraday coefficients of variation for the high, medium, and low concentrations ranged from 1.5% to 10.7% for all four analytes. The lower limit of detection for all four analytes was no less than 75 ng/mL.

Results

Subjects and Toxicity. Twenty-seven healthy volunteers, 9 males and 22 females, with a mean age of 25.2 ± 8.4 years (range, 19-61 years) were recruited from April through September 2005. Nearly one half (N = 13, 48.1%) of the participants were Caucasian; one third were Asian (N = 9, 33.3%); and more than 7% (N = 2) reported being of Hispanic ethnicity. All toxicities reported are shown in Table 1. No toxicities greater than the National Cancer Institute Common Toxicity Criteria (version 2.0) grade 1 were reported. The major treatment-associated toxicities were minor gastrointestinal upsets, including eructation, heartburn, and indigestion.

Detection of 6-Gingerol, 8-Gingerol, 10-Gingerol, and 6-Shogaol and Conjugate Metabolites in Plasma Samples. All plasma samples were analyzed both with and without incubation and deconjugating enzymes. No free 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol were detected in the plasma of any participants. Consequently, the subsequent results refer exclusively to 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol conjugates that were quantified after the treatment of samples with β-glucuronidase and sulfatase. The pharmacokinetic parameters (Cmax, Tmax, and area under the curve) of the three highest doses of 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol conjugates are presented in Table 2. The AUC was calculated by extrapolating to the last observed time point with measurable concentrations. The t1/2 was only calculated for the 2.0-g dose for all conjugates. The 2.0-g dose was the only dose in which the decline of plasma concentration was consistent enough to allow for half-life estimation. The Tmax ranged from 30 to 80 minutes for all four conjugates at all doses. Gingerol and shogaol conjugates were completely eliminated from the plasma at the 4-hour time point although one participant who received a 2.0-g dose still had detectable 6-gingerol conjugates at 8 hours after ingestion. Only 6-gingerol conjugates were detectable below the 1.0-g dose. The area under the curve for the 6-gingerol conjugates were 2.8 ± 2.5 and 5.3(±3.0) µg/mL for the 250-mg and 500-mg dose, respectively. The Cmax for the lower 6-gingerol doses were 0.3 (±0.3) and 0.4 (±0.23) µg/mL.

The concentrations (mean ± SD) of the three highest doses of 6-gingerol glucoronides and sulfates at Tmax are presented in Table 3. Little to no conjugate metabolites...
were detected below the 1.0-g dose and are thus not presented in Table 3. No 8-gingerol, 10-gingerol, and 6-shogaol sulfates were detectable even at doses of 1.0 g and above. In addition, glucuronide metabolites were negligible for 8-gingerol, 10-gingerol, and 6-shogaol except for the 2.0-g dose, in which 0.30 ± 0.33 μg/mL of 8-gingerol, 0.18 ± 0.26 μg/mL of 10-gingerol, and 0.14 ± 0.25 μg/mL of 6-shogaol were detectable. The concentrations of conjugate metabolites were similar between the separate and mixed assays at all three dose levels (1.0, 1.5, and 2.0 g) for 8-gingerol, 10-gingerol, and 6-shogaol, except for 10-gingerol, in which there were more mixed conjugates compared with separate conjugates. For instance, at the 2.0-g dose, there was 0.18 ± 0.20 μg/mL for the separate assay versus 0.36 ± 0.26 μg/mL for the mixed assay.

The sulfate conjugates were below the detectable assay limits for all participants at any dose for both 10-gingerol and 6-shogaol, and only one participant at the 2.0-g dose for 8-gingerol had detectable sulfate conjugates. Six-gingerol sulfate conjugates were not detectable below the 1.0-g dose, with only one third of the participants at 1.0 g and two thirds at the 1.5-g and 2.0-g dose having detectable concentrations. The glucuronide conjugates were similar, with only a few participants having detectable concentrations of any metabolites below 1.0 g of ginger.

The relative proportion of glucuronides to sulfates is presented in Table 3. The relative amount of glucuronide conjugates was higher than the amount of sulfate conjugates at each participant’s observed Tmax by 5% to 93% for 6-gingerol, 71% to 100% for 8-gingerol, 4% to 67% for 10-gingerol, and 0% to 100% for 6-shogaol.

Discussion

The results indicate that no free 6-gingerol, 8-gingerol, 10-gingerol, or 6-shogaol was detectable in the plasma within the dose range investigated. All four analytes were, however, quickly absorbed after p.o. dosing and can be detected as glucuronide and sulfate conjugates.

Table 2. 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol pharmacokinetic parameters estimated from the raw data

<table>
<thead>
<tr>
<th>6-gingerol</th>
<th>Dose = 1,000 mg (N = 6)</th>
<th>Dose = 1,500 mg (N = 3)</th>
<th>Dose = 2,000 mg (N = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Min (max)</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>AUC*</td>
<td>12.6 (6.4)</td>
<td>3.6 (20.5)</td>
<td>75.6 (110.3)</td>
</tr>
<tr>
<td>Cmax*</td>
<td>0.4 (0.2)</td>
<td>0.2 (0.7)</td>
<td>1.69 (2.31)</td>
</tr>
<tr>
<td>t1/2*</td>
<td>55.0 (7.7)</td>
<td>45.0 (60.0)</td>
<td>60.0 (0.0)</td>
</tr>
<tr>
<td>tmax</td>
<td>52.5 (8.7)</td>
<td>45.0 (60.0)</td>
<td>60.0 (0.0)</td>
</tr>
<tr>
<td>8-gingerol</td>
<td>AUC</td>
<td>2.1 (2.2)</td>
<td>0 (4.5)</td>
</tr>
<tr>
<td>Cmax</td>
<td>0.1 (0.1)</td>
<td>0 (0.2)</td>
<td>0.1 (0.1)</td>
</tr>
<tr>
<td>t1/2</td>
<td>52.5 (8.7)</td>
<td>45.0 (60.0)</td>
<td>60.0 (0.0)</td>
</tr>
<tr>
<td>tmax</td>
<td>10-gingerol</td>
<td>AUC</td>
<td>2.9 (3.2)</td>
</tr>
<tr>
<td>Cmax</td>
<td>0.1 (0.1)</td>
<td>0 (0.4)</td>
<td>0.1 (0.02)</td>
</tr>
<tr>
<td>t1/2</td>
<td>60.0 (0.0)</td>
<td>60.0 (60.0)</td>
<td>80.0 (34.6)</td>
</tr>
<tr>
<td>tmax</td>
<td>6-shogaol</td>
<td>AUC</td>
<td>0.8 (1.5)</td>
</tr>
<tr>
<td>Cmax</td>
<td>0.1 (0.1)</td>
<td>0 (0.1)</td>
<td>0.04 (0.08)</td>
</tr>
<tr>
<td>t1/2</td>
<td>55.0 (8.7)</td>
<td>45.0 (60.0)</td>
<td>60.0 (0.0)</td>
</tr>
<tr>
<td>tmax</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: AUC was determined by the trapezoid rule; t1/2 was determined by means of linear interpolation.
*Only AUC and Cmax are recorded as μg/mL.
**t1/2 and tmax are recorded in minutes.
†These results are based on detectable free gingerols and shogaols after incubation with β-glucuronidase and sulfatase and thus represent combined conjugates.
in the serum. The majority of the metabolites were glucuronide conjugates. No sulfate conjugates were detected, with the exception of 6-gingerol, in which only at the highest dose was any significant amount of sulfates detected and roughly on third of the conjugates were sulfates. Further, the metabolites seemed to be either glucuronides or sulfates and not mixed conjugates although, once again, 6-gingerol seemed to be an exception with more mixed conjugates seen at lower doses. These results are similar with those observed with p.o. dosing of 6-gingerol in rats, in which no free 6-gingerol was found at any time point in the urine or bile, but (5)-[6]-gingerol-4-O-β-glucuronide was present in the bile, and no sulfate conjugates were detectable (38). Further, Metzler and colleagues found that human intestinal microsomes and hepatic microsomes fortified with UDP-glucuronyl transferase enzymes only formed glucuronides of 6-gingerol and that UGT1A1, UGT1A3, and UGT2B7 (these are expressed in both the liver and intestinal mucosa) were responsible for the production of the glucuronides (40). Currently, however, it is unclear if gingerols/shogaols are conjugated to glucuronides in the intestinal mucosa, liver or both, and if free or already conjugated gingerols and shogaols reach the liver and are further conjugated with sulfate to form glucuronide/sulfate conjugates there. Future research will need to be conducted to determine the relative importance of UDP-glucuronyl transferase activity in the liver compared with that in the intestinal mucosa as well as the contribution of liver sulfate enzymes.

Ginger conjugates began to appear 30 minutes after p.o. dosing, reaching their $T_{\text{max}}$ between 45 minutes to 120 minutes, with elimination half-lives ranging from 75 minutes to 120 minutes at the 2.0-g dose. These results differ from both the i.v. bolus and p.o. studies conducted in rats. I.v. bolus studies in rats found that free 6-gingerol is rapidly cleared from the plasma with a terminal half-life ranging from 7.23 minutes to 8.5 minutes and that 6-gingerol is not detectable after 30 minutes (36). Whereas a single p.o. dosing of 6-gingerol in rats resulted in the rapid appearance of glucuronide conjugate, it did not reach its maximum concentration until after 12 hours and was detectable for at least 60 hours after ingestion (38). The difference between the present study and the i.v. injection of 6-gingerol would seem to be primarily due to the method of delivery. I.v. 6-gingerol would bypass being metabolized by the gut bacteria, the intestinal epithelium, or the liver and, thus, in its nonconjugated form that is detectable quickly after injection. Also, it seems that i.v. free 6-gingerol was much more rapidly cleared from the system compared with conjugate metabolites. The difference in time to maximum concentration and elimination between the p.o.–administered 6-gingerol in rats and in humans could be due to differences between species or differences in dose. The dose of 6-gingerol given to the rats was approximately equivalent to a human dose of 583.3 mg of 6-gingerol.

This is much higher than 43.04 mg, the maximum amount of 6-gingerol the participants in our study were given.

The maximum serum concentrations of the ginger analytes were reached at either the 1.5-g or 2.0-g dose and were 1.69 μg/mL for 6-gingerol, 0.23 μg/mL for 8-gingerol, 0.53 μg/mL for 10-gingerol, and 0.15 μg/mL for 6-shogaol. The lack of free gingerols and shogaols and the low concentration of ginger metabolites in the serum can be used to assess the potential clinical relevance of the reported in vitro research employing these ginger components. For instance, in prostate cancer cell lines, 14.72 μg/mL (50 μM) of 6-gingerol was needed to inhibit MKP5, a key mediator of pro-inflammatory pathways and cancer cell growth in prostate cells (19). In another study, 6-shogaol was found to induce apoptosis, autophagocytosis, and growth inhibition in ovarian cancer cells at 2.21 μg/mL (7.5 μmol/L; ref. 20). All of these in vitro studies required higher concentrations of free ginger constituents than those found in the serum in this study, putting the clinical validity of these and similar studies in question. However, gingerols and shogaols may reach higher serum concentrations in target tissue compared with the serum (e.g., gut). Ginger conjugates may also be as or more biologically active compared with parent compound (40). Clearly, further research is needed to answer these questions and determine the cancer prevention relevance of ginger.

In this trial, no serious adverse effects were reported after ingesting up to 2.0 g of standardized ginger extract. All toxicities reported were mild and correspond to grade 1 of the National Cancer Institute common toxicity scale (Table 1). Consistent with previous clinical research, the majority of the adverse events were transient gastrointestinal upsets, such as gas and bloating. Where- as the small size of this trial precludes any formal safety endpoint analysis and statistical certainty of safety, the safety profile observed here is consistent with previous clinical and preclinical data (6).

With the exception of 6-gingerol, the analytes were not well absorbed, with no detectable conjugate metabolites below the 1.0-g ginger-extract dose. The lack of detectable analytes below the 1.0-g dose was likely due to the low amount of individual analytes in the ginger extract.
with only 21.52 mg of 6-gingerol, 7.20 mg of 8-gingerol, 16.76 mg of 10-gingerol, and 3.68 mg of 6-shogaol in the 1.0-g dose. Another possible reason for the lack of detectable analytes could be the lack of stability of gingerols and shogaols in plasma during storage and analysis. This explanation seems unlikely because 6-gingerols seem stable in conditions similar to the ones used in our analysis although the stability of 6-gingerols was only determined in aqueous solution and not in plasma (42). Because of low levels of absorption, the participants receiving the highest dose did not have adequate detectable concentrations after Cmax to reliably calculate the elimination half-life. Consequently, no pharmacokinetic model was able to be constructed, and the pharmacokinetic parameters are based on noncompartment analysis with an elimination half-life only presented for the 2.0-g dose. Even at the 2.0-g dose, the half-life needs to be interpreted with caution because estimates were based on a limited data set.

Future studies should focus on obtaining information for conducting both single-dose and multi-dose pharmacokinetic modeling. The information gained from pharmacokinetic modeling could then be used to optimize the dose and dose regimen in clinical phase-1 or phase-2 trials and to enhance the delivery of the ginger extract. Pharmacokinetic models could be developed by enriching the number of serum samples taken between baseline and 4 hours after p.o. administration, with particular emphasis on the serum concentrations after 60 minutes when, on average, time to maximum concentration is reached. In addition, higher doses of pungent gingerol constituents could be administered, or more sensitive analytic methods for detecting gingerol and shogaol conjugates in the picogram concentration could be developed. All three of these techniques would help to better describe pharmacokinetic parameters and ensure the calculation of accurate elimination half-lives. Multi-dose pharmacokinetic parameters and safety also need to be investigated to explore the possibility of any toxicity or tolerability issues from longer-term dosing. Pharmacokinetic information studies in populations that are likely to be the target for ginger therapeutically, such as people at high risk for colorectal cancer and older populations with common comorbidities, also need to be conducted. Further work is also needed to determine the range of gingerol and shogaol metabolites found in humans and the activity of these metabolites to modulate important cancer markers, such as NFκB or a variety of inflammatory eicosanoids (i.e., prostanoids and/or prostaglandin E2).

In summary, the main pungent constituents of ginger root, 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol, are quickly absorbed and detected in the serum as glucuronide and sulfate conjugates, with the majority detected as glucuronide metabolites. These constituents, at concentrations normally found in ginger root (0.5%–2.5%), are detectable in the serum starting at a 1.0-g dose with the exception of 6-gingerol, which is detectable at a 250-mg dose with maximum concentrations ranging from 0.1 μg/mL to 1.7 μg/mL.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Pure Encapsulations® (Sudbury, Massachusetts) for donating the ginger extract.

References


Brief report: An open-label study of the neurosteroid pregnenolone in adults with autism spectrum disorder.
J Autism Dev Disord. 2014
Brief report: An open-label study of the neurosteroid pregnenolone in adults with autism spectrum disorder.

J Autism Dev Disord. 2014

Lawrence K. Fung · Robin A. Libove · Jennifer Phillips · Francois Haddad · Antonio Y. Hardan

Abstract The objective of this study was to assess the tolerability and efficacy of pregnenolone in reducing irritability in adults with autism spectrum disorder (ASD). This was a pilot, open-label, 12-week trial that included twelve subjects with a mean age of 22.5 ± 5.8 years. Two participants dropped out of the study due to reasons unrelated to adverse effects. Pregnenolone yielded a statistically significant improvement in the primary measure, Aberrant Behavior Checklist (ABC)-Irritability [from 17.4 ± 7.4 at baseline to 11.2 ± 7.0 at 12 weeks (p = 0.028)]. Secondary measures were not statistically significant with the exception of ABC-lethargy (p = 0.046) and total Short Sensory Profile score (p = 0.009). No significant vital sign changes occurred during this study. Pregnenolone was not associated with any severe side effects. Single episodes of tiredness, diarrhea and depressive affect that could be related to pregnenolone were reported. Overall, pregnenolone was modestly effective and well-tolerated in individuals with ASD.

Keywords Autism spectrum disorder · Pregnenolone · Neurosteroids · Irritability · Open-label trial

Introduction

Autism spectrum disorder (ASD) is characterized by deficits in social communication and interactions, stereotypic behaviors, and restricted interests (APA 2013). In addition to these core symptoms, persons with ASD often suffer from irritability, which may manifest as tantrums, self-injury, and aggressive behaviors toward others. These symptoms often cause significant challenges to individuals with ASD and their families. Behavioral interventions are usually the first treatments for the associated behaviors, but when not effective and especially when the symptoms cause significant impairment in functioning, pharmacologic treatments for irritability may be considered. Currently, risperidone and aripiprazole are the only medications approved by the US Food and Drug Administration for the treatment of irritability in individuals with ASD. These atypical antipsychotic medications are effective in reducing irritability. However, adverse effects such as metabolic syndrome, extrapyramidal symptoms, and tardive dyskinesia can be devastating to the patients and their families. Therefore, effective medications with more tolerable side effect profiles will be more desirable.

One proposed model of ASD suggests that at least some forms of this disorder are a result of an altered ratio of excitation/inhibition (E/I) in key neural systems (Rubenstein and Merzenich 2003). Brain neurotransmission pathways based on gamma aminobutyric acid (GABA) are known to be inhibitory, while the pathways based on glutamate are known to be excitatory (Kandel et al. 2013). Mounting evidence from animal models and human studies support this hypothesis on E/I imbalance. A meta-analysis of transgenic mouse models of ASD revealed that the number of parvalbumin (PV)-positive GABAergic interneurons was reduced in the neocortex, suggesting that PV-
circuit disruption may be relevant in the pathogenesis and pathophysiology of ASD (Gogolla et al. 2009). Further evidence of abnormalities of the GABAergic system in ASD was provided in postmortem and neuroimaging studies. Reduced expressions of specific subunits of GABA_\alpha_2 as well as GABA_\beta_2 receptors were found in the postmortem brains of adults with ASD (Fatemi et al. 2010, 2014). Additionally, a recent preliminary positron emission tomography (PET) study showed lower densities of \( \alpha_1 \) and \( \alpha_5 \)-containing GABA_\alpha_ receptors in the brains globally (but especially amygdala and nucleus accumbens) of high-functioning individuals with ASD when compared with healthy controls (Mendez et al. 2012). Collectively, modulation of the GABAergic pathways is predicted to change the E/I ratio and therefore may be considered as a strategy for treating irritability in individuals with ASD. In addition to the GABAergic pathways, emerging evidence has shown abnormalities of the glutamatergic pathways in ASD (Gai et al. 2012; O’Roak et al. 2011) as well. Given the alterations of the GABAergic and glutamatergic neurotransmission systems, we speculate that substances, which can modulate these systems may be relevant in the treatment of ASD.

Neurosteroids are steroids synthesized within the central nervous system as well as in other steroidogenic organs/tissues. In contrary with the classical actions through intracellular receptors, extensive evidence has established that neurosteroids can exert rapid, potent actions at the cell membrane via allosteric interactions with the GABA_\alpha_ receptor. Pregnenolone is a naturally occurring neurosteroid directly metabolized from cholesterol and the precursor of virtually all neurosteroids. This hormone has been used as a dietary supplement. When orally administered in humans, pregnenolone is converted to multiple metabolites. Among them, the most abundant metabolites are allopregnanolone and pregnenolone sulfate (Sripada et al. 2013). In contrast, cortisol level was shown to remain the same after administration of pregnenolone (Marx et al. 2009). Allopregnanolone has been shown to regulate GABA_\alpha_ receptors through two discrete transmembrane sites (Hosie et al. 2006) and positively modulate GABA_\alpha_ receptors (Majewska et al. 1986). The sulfated form of pregnenolone was recently found to stimulate the trafficking of N-methyl-D-aspartate (NMDA) receptors to neuronal surface (Kostakis et al. 2013). The pharmacologic actions of allopregnanolone and pregnenolone sulfate may potentially be relevant mechanisms of action in relieving symptoms in ASD.

Oral administration of pregnenolone was recently shown to be associated with enhanced activation of neurocircuits controlling emotion regulation (Sripada et al. 2013). Compared with placebo, allopregnanolone (as measured after taking pregnenolone) was associated in healthy adults with increased activity in the dorsal medial prefrontal cortex and enhanced connectivity between the amygdala and dorsal medial prefrontal cortex, an effect that was associated with reduced self-reported anxiety. Pregnenolone was shown to be superior over placebo treatments in at least three randomized, placebo-controlled, double-blind trials of psychiatric disorders (Marx et al. 2009; Osuji et al. 2010; Ritsner et al. 2010). In patients with co-morbid major depressive and substance disorders, pregnenolone reduced the Hamilton rating scale for depression (HRSD) scores in a post hoc analysis of completers (Osuji et al. 2010). Pregnenolone was also shown to improve negative symptoms in patients with schizophrenia (Marx et al. 2009). Finally, a low dose of pregnenolone given adjunctively to patients with schizophrenia and schizoaffective disorder ameliorated positive symptoms and resulted in improvement in attention and working memory (Ritsner et al. 2010). Pregnenolone was also found to be well tolerated by participants in all reported studies to date.

In light of the above-discussed evidence, we completed an open-label, 12-week trial of pregnenolone, a dietary supplement, in adults with ASD with the objectives of examining its effect on irritability and to assess its safety and tolerability in this population.

**Methods**

This was a 12-week, open-label study of pregnenolone in twelve adults with ASD conducted in the authors’ home institution between November 2011 and September 2013. Informed consent was signed by the legal guardians and assent was obtained from participants when possible. Subjects were then screened and inclusion and exclusion criteria were assessed. No changes in eligibility criteria were applied throughout the study. This investigation was approved by the Institutional Review Board at the authors’ home institution. An investigational new drug application (#109191) was filed with the Food and Drug Administration. The full trial protocol is available upon request.

**Participants**

Fifteen individuals signed consent forms for this study, but only 12 met inclusion/exclusion criteria and were included in this prospective study. Subjects with and without intellectual disability were enrolled. Inclusion criteria consisted of the following: (a) outpatients 18–45 years of age; (b) males and females who are physically healthy; (c) diagnosis of autistic disorder based on expert clinical evaluation and DSM-IV-TR criteria, and confirmed using the Autism Diagnostic Interview-Revised (Lord et al. 1994), and the Autism Diagnostic Observation Schedule.
and routine urinalysis) were performed at baseline and at complete blood count with differential, cholesterol panel, EKG and laboratory tests (complete metabolic panel, were monitored for adverse events in each visit. In addition, EKG and laboratory tests (complete metabolic panel, complete blood count with differential, cholesterol panel, and routine urinalysis) were performed at baseline and at the end of treatment phase at Week 12. Urine toxicology screen and pregnancy test (for female subjects) were performed at screening phase.

Briefly, the ABC is a standardized scale, comprising 58 items, for assessing problem behavior in subjects with intellectual disability and developmental disabilities (Aman et al. 1985). The checklist was empirically derived from ratings on approximately 1,000 subjects, and the items resolve into five subscales: irritability, lethargy/social withdrawal, stereotypic behavior, hyperactivity, and inappropriate speech. High ABC scores indicate more severe behavioral symptoms. The SSP is a 38-item parent report questionnaire that evaluates sensory abnormalities and compares to available normative data (Tomchek and Dunn 2007). The items in SSP are written such that low scores reflect undesirable and abnormal behaviors. The SRS is a 65-item parent report questionnaire designed to measure the severity of autism spectrum symptoms as they occur in natural social settings (Constantino et al. 2003). High scores in SRS indicate more severe behavioral symptoms. VABS is a 433-item parent report questionnaire designed to assess personal and social skills needed for everyday living (Sparrow et al. 2005). Low scores in VABS suggest lower adaptive abilities.

Pharmacological Intervention

Pregnenolone capsules were obtained from Pure Encapsulations (Sudbury, MA, USA). The capsules combined a 99% purity pregnenolone powder with hypo-allergenic plant fiber (cellulose) in a vegetarian capsule (derived from cellulose and water). Pregnenolone was initiated at a dose of 50 mg twice daily for the first 2 weeks and was increased by 50 mg twice daily every 2 weeks until reaching the maximal dosage of 250 mg twice daily from weeks 9 to 12. If subjects could not tolerate a specific dose, s/he would be maintained at the highest tolerated dose. At the end of this initial phase, pregnenolone was decreased by 50 mg twice a day every 3 days until it was discontinued.

Assessments

Primary outcome measures included the ABC-I. Secondary measures included the other subscales of ABC, Short Sensory Profile (SSP), Social Responsiveness Scale (SRS), and Vineland Adaptive Behavior Scale (VABS). The ABC, SSP, SRS, VABS were administered at baseline, at the end of the 12-week treatment. Participants were also invited back for a follow-up visit 4 weeks after the end of the trial to monitor the effect of tapering and discontinuation of the medication. On an exploratory basis, ABC was also monitored at 2, 4, 6, 8 and 10 weeks. Vital signs and Dosage Record and Treatment Emergent Symptom Scale (DOTES) were monitored for adverse events in each visit. In addition, EKG and laboratory tests (complete metabolic panel, complete blood count with differential, cholesterol panel, and routine urinalysis) were performed at baseline and at

Statistical Analyses

Data analysis was conducted with SPSS 19 (SPSS Inc., Chicago, IL, USA). Paired, 2-tailed student t-tests were performed to compare primary and secondary outcome measures between baseline and at 12 weeks using last observation carried forward approach. Differences were considered significant with \( p < 0.05 \). Effect sizes (Cohen’s d) for statistically significant findings were calculated by the following equation:

\[
\text{Cohen's d} = \frac{\text{Measure}_{\text{end}} - \text{Measure}_{\text{baseline}}}{\text{Measure}_{\text{stdev,pool}}}
\]

where Measure\(_{\text{end}}\) is the mean of the outcome measure at the end of treatment; Measure\(_{\text{baseline}}\) is the mean of the outcome measure at baseline; and Measure\(_{\text{stdev,pool}}\) is the pooled standard deviation.

Additional exploratory analyses were performed on the primary outcome measure (ABC-I) and included repeated measures ANOVA in analyzing longitudinal data in the treatment phase and a paired, 2-tailed student \( t \) test comparing week 12 and the follow-up visit after pregnenolone was discontinued Table 1.

Results

Ten men and two women with ASD (mean age 22.5 ± 5.8 years; range 18.1–35.5 years; 9 Caucasians and
3 Asians) met the study criteria for inclusion in this open-label study. One participant failed inclusion criteria due to incidental findings on the baseline EKG (right bundle branch block and possible right ventricular hypertrophy). All participants were taking psychotropic medications with the exception of one subject. Concomitant medications were maintained at the same dosage throughout the study and 8 participants were on second-generation antipsychotics, 3 on selective serotonin reuptake inhibitors, 4 on mood stabilizers (including lithium, valproic acid, carbamazepine), 1 on buspirone, 1 on trazodone, 1 on diazepam, 1 on zolpidem, 2 on melatonin, and 1 on benadryl.

**Treatment Response**

Pregnenolone yielded a statistically significant improvement in the primary measure, ABC-I at 12 weeks [from 17.4 ± 7.4 at baseline to 11.2 ± 7.0 at 12 weeks (p = 0.028, df = 11, t = 2.5); d = −0.86]. Secondary measures were not statistically significant with the exception of ABC-lethargy/social withdrawal (ABC-L/SW; p = 0.046, df = 11, t = 2.3; d = −0.64) and total SSP score (p = 0.009, df = 10, t = 3.2; d = 0.53). Exploratory repeated measures analysis of the longitudinal ABC-I data revealed a trend toward statistical significance (Greenhouse-Geisser: F = 2.17; p = 0.089). No difference in the primary outcome measure (ABC-I) was observed between week 12 follow-up visit and 4 weeks after the end of the treatment phase (week 12: 11.9 ± 6.8; week 16: 12.6 ± 7.7; t = −0.606, df = 10, p = 0.558).

**Safety Measures and Adverse Effects**

During the 12-week treatment period, two participants dropped out of the study. The parents of one of the participants decided to withdraw from the study after 4 weeks (day 29) since patient was getting frustrated from taking too many pills twice a day. Another participant exhibited worsening of baseline behaviors during week 8, which led mother to withdraw him from the study at day 58. The decrease and discontinuation of pregnenolone did not lead to the amelioration of behaviors, which led to the adjustment of the baseline medications that he was taking.

Pregnenolone was not associated with any severe adverse effects. Single episodes of tiredness (n = 1), diarrhea (n = 1), and depressive affect (n = 1) that could possibly be related to pregnenolone were reported. A few other adverse events with remote chance to be related to the medication were reported: increased excitement/agitation (n = 3), sleep problems (n = 1), drowsiness (n = 1), anorexia/decreased appetite (n = 2), increased motor activity (n = 1), sweating (n = 1), constipation (n = 1), diarrhea (n = 1), tremor (n = 1), and depressive affect (n = 1). No significant vital sign or EKG changes occurred in any study participants (Table 2). No abnormal laboratory tests were caused by pregnenolone.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Clinical measures</th>
<th>Baseline</th>
<th>Week 12</th>
<th>Paired t test</th>
<th>Effect size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Aberrant behavioral checklist</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABC-Irritability</td>
<td>17.4</td>
<td>7.4</td>
<td>11.2</td>
<td>7.0</td>
<td>2.5</td>
</tr>
<tr>
<td>ABC-lethargy/social withdrawal</td>
<td>18.1</td>
<td>8.0</td>
<td>12.8</td>
<td>8.7</td>
<td>2.3</td>
</tr>
<tr>
<td>ABC-stereotypy</td>
<td>9.8</td>
<td>5.5</td>
<td>8.7</td>
<td>6.5</td>
<td>0.7</td>
</tr>
<tr>
<td>ABC-hyperactivity</td>
<td>20.5</td>
<td>16.1</td>
<td>16.1</td>
<td>8.9</td>
<td>1.8</td>
</tr>
<tr>
<td>ABC-inappropriate speech</td>
<td>5.8</td>
<td>4.3</td>
<td>4.8</td>
<td>4.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Short sensory profile—total score</td>
<td>137.7</td>
<td>21.5</td>
<td>147.6</td>
<td>15.3</td>
<td>−3.2</td>
</tr>
<tr>
<td>Social responsiveness scale—total score</td>
<td>84.9</td>
<td>8.1</td>
<td>84.5</td>
<td>9.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Vinelandb—adaptive behavior composite score</td>
<td>37.3</td>
<td>13.1</td>
<td>42.9</td>
<td>16.5</td>
<td>−1.3</td>
</tr>
</tbody>
</table>

| Table 2 | Vital signs at baseline and end of pregnenolone treatment at week 12 | | |
| | Clinical measures | Baseline | End of treatment | Paired t test | |
| | | N = 12 | N = 12 | t | p |
| | | Mean | SD | Mean | SD | |
| Systolic blood pressure (sitting) | 119 | 15 | 121 | 11 | −0.663 | 0.521 |
| Diastolic blood pressure (sitting) | 74 | 9 | 76 | 6 | −0.672 | 0.515 |
| Pulse (sitting) | 85 | 13 | 83 | 14 | 0.5 | 0.627 |
| Systolic blood pressure (standing) | 117 | 14 | 120 | 11 | −0.734 | 0.478 |
| Diastolic blood pressure (standing) | 75 | 7 | 77 | 6 | −1.081 | 0.303 |
| Pulse (standing) | 92 | 11 | 89 | 17 | 0.887 | 0.394 |
| Weight (pounds) | 179 | 74 | 180 | 76 | −0.223 | 0.828 |
Discussion

This hypothesis-generating study represents the first attempt to provide preliminary support for the use of pregnenolone, a neurosteroid and an oral supplement, in the treatment irritability in individuals with ASD. Irritability is a non-specific mood state, which is postulated to be controlled by neural processes responsible for “top down inhibition” and “bottoms-up drive” (Siever 2008). Generally, the prefrontal cortex (PFC) and anterior cingulate cortex (ACC) are thought to be the neural substrate for “top down inhibition”, whereas the amygdala and insula are often associated with “bottoms-up drive”. When these regions are dysfunctional, it is suggested that affected individuals will exhibit irritability more severely and readily. In typically developing individuals, cognitive control was shown to be associated with optimal functional connectivity between dorsolateral PFC and parietal cortex; in contrast, in ASD, cognitive control was shown to be controlled by the ventrolateral PFC and ACC (Solomon et al. 2013). Compared to typically developing controls, participants with ASD were shown to have reduced functional connectivity between the insula and specific brain regions involved in emotional processing (e.g. amygdala) and sensory processing (e.g. somatosensory cortex) (Ebisch et al. 2011). Furthermore, emerging evidence has shown that alterations of the GABAergic system in ASD were also present in some of the same areas responsible for “top down inhibition” and “bottoms-up drive”. For example, decreased GABA_A receptors have been found in the ACC of postmortem brains of persons with ASD (Oblak et al. 2009). As mentioned, a recent pilot PET study showed lower densities of α1 and α5-containing GABA_A receptors in the amygdala of high-functioning individuals with ASD when compared with healthy controls (Mendez et al. 2012). Given the above evidence, pregnenolone might exert its pharmacologic effects through its GABAergic metabolite allopregnanolone. Interestingly, a synthetic analog of allopregnanolone, ganaxolone, is currently being tested for treating children with fragile X syndrome, a syndromic form of ASD.

The effect size for pregnenolone at 12 weeks on ABC-I in adults with ASD in the current study (−0.86) is large, but is generally lower than the effect sizes for atypical antipsychotics in early open-label studies in ASD. For example, in an 8-week open-label study of paliperidone in 25 adolescents and young adults with ASD, the effect size for the medication on ABC-I was −2.2 (Stigler et al. 2012). The outcomes for open-label trials of olanzapine in children and adolescents with ASD were mixed [d = −2.1 (Fido and Al-Saad 2008) and −0.4 (Kenmer et al. 2002) on ABC-I]. In one of risperidone’s earliest investigations in the treatment of disruptive behaviors in children with ASD, 12-week treatment resulted in effect sizes of −1.0 and −0.8 in the items “Irritable” and “Aggression” in the Clinician-Rated Visual Analog Scales (McDougle et al. 1997). The effect size for aripiprazole at 14 weeks on ABC-I in children and adolescents with ASD was very large [−2.8 (Stigler et al. 2009)]. Overall, when comparing open-label studies, pregnenolone appears to be less potent than atypical antipsychotics in the treatment of irritability and associated behaviors in ASD.

Pregnenolone’s main active metabolite, allopregnanolone, is a positive modifier of the GABA_A receptor. As mentioned earlier, individuals with ASD were shown to have lower GABA_A receptor density in the amygdala (Mendez et al. 2012). As social processes are at least partially moderated by the amygdala, increase in allopregnanolone levels in the brain after administration of pregnenolone can potentially increase the GABAergic tone in the amygdala. Interestingly, this hypothesis is consistent with the improvement in ABC-L/SW score at the end of pregnenolone treatment. However, the improvement in ABC-L/SW score was inconsistent with the unchanged SRS total score. This discrepancy is possibly related to the fact that the two rating scales assess different social domains, or simply, changes observed on the ABC-withdrawal are false positive. Hence this observation should be interpreted with caution.

Pregnenolone was overall well tolerated, with few side effects and no severe adverse effects reported in this study. Additionally, due to pregnenolone sulfate’s known role in activating TRPM3 (Naylor et al. 2010), a subtype of transient receptor potential (TRP) channels expressed in the brain and kidneys, we monitored cardiovascular effects carefully by measuring vital signs in all subjects and EKGS were obtained at baseline and at the end of the trial. During this 12-week study, we did not find any significant changes in blood pressure, pulse, and body weight and no EKG changes were observed. These observations suggest that pregnenolone is relatively safe in adults with ASD. However, further studies are needed before final conclusions can be made.

Findings from this study should be cautiously interpreted in light of several limitations. First, this is an open-label trial with a very small sample size. Placebo response is commonly observed in open-label trials. Second, although pregnenolone is known to metabolize mainly to allopregnanolone and pregnenolone sulfate [and not other downstream metabolites such as cortisol; (Marx et al. 2009)], no measures of plasma or salivary concentrations of these metabolites were completed in this study. Third, although the ABC-I at 12 weeks was significantly lower than baseline value, our exploratory repeated measures ANOVA analyses did not reach statistical significance. The discrepancy is likely due to the small sample size. Fourth,
inclusion criteria did not include a cut-off for ABC-I. Finally, no changes in ABC-I were observed after pregnenolone was discontinued. This might suggest that improvement observed in the study might be unrelated to the study medication. It might also be related to a long-lasting effect of pregnenolone and a longer follow-up period might be necessary.

In conclusion, in this pilot study, pregnenolone, an oral supplement, was modestly effective and was overall safe and well tolerated in individuals with ASD. More importantly, this investigation has generated potentially important hypotheses for the treatment of ASD. It supports further studies in exploring pregnenolone’s effects on reducing irritability, improving social functioning (a core symptom of ASD), and attenuating sensory abnormalities. As the currently FDA-approved medications for the treatment of irritability in ASD have significant serious adverse side effects, pregnenolone may be a potential alternative due to its milder side effect profile. Furthermore, social and sensory deficits are among the core features of ASD and no effective treatment is currently available. Therefore, randomized large trials of pregnenolone and its associated neurosteroids targeting irritability, abnormal social interactions, and/or sensory aberrations may potentially lead to novel treatments for these symptoms in ASD.

Acknowledgments This study was supported by a grant from the Escher Family Fund at the Silicon Valley Community Foundation to AYH and Mosher Fund to LKF. LKF is a recipient of the Ruth L. Kirschstein Individual Postdoctoral National Research Service Award. The authors would like to thank Pure Encapsulations (Sudbury, MA, USA) for donating the pregnenolone capsules.

Conflict of interest Over the last 3 years, Dr. A.Y. Hardan has received research support and honorarium for consulting from the following companies: Bristol-Myers Squibb, Roche, Forest, and IntegraGen. Drs. L. K. Fung, J. Phillips, F. Haddad and Ms. R. A. Libove did not report any conflict.

References


