Journal of Aging Research & Clinical Practice©
Volume 1, Number 3, 2012

ANTIOXIDANT EFFECTS OF A NUTRITIONAL SUPPLEMENT CONTAINING POLYPHENOLS AND MICRONUTRIENTS IN POSTMENOPAUSAL WOMEN: A RANDOMIZED CONTROLLED STUDY

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Abstract: *Background:* Oxidative stress is an important factor in the development of osteoporosis. Antioxidants counteract the damaging effect of oxidative stress, which may reduce the risk of osteoporosis. Nutritional supplements, such as greens+TM and greens+ bone builder™ that contain water-soluble polyphenols and other micronutrients beneficial for bone health, are of recent interest as complementary strategies in the management of osteoporosis. *Objective:* Clinically evaluate the antioxidant properties of greens+ bone builder™ in postmenopausal women. *Design:* Forty-seven postmenopausal women, 50-60 years old were recruited for a ten-week clinical study. During week 1, participants recorded their baseline food intake. During week 2, the participants refrained from consuming polyphenol-rich foods, beverages and supplements. The participants were then randomized to either Treatment group consuming 1 scoop (equivalent to ¼ cup) daily of greens+ bone builder™ (N=23) or Placebo (N=24) group for a period of 8 weeks. Blood samples were collected at 0, 4 and 8 weeks of supplementation, processed and assayed for serum total antioxidant capacity (TAC), lipid peroxidation and protein oxidation as markers of oxidative stress. *Results:* Statistical analysis showed that after 4 and 8 weeks, the Treatment group significantly increased their serum total antioxidant capacity and decreased in lipid peroxidation and protein oxidation while the Placebo group showed no significant changes. These were also significantly different from those of the Placebo group. *Conclusions:* Results suggest that a daily supplementation with greens+ bone builder™ may be important in reducing oxidative damage, thus reducing the risk of osteoporosis in postmenopausal women.

Key words: Reactive oxygen species, oxidative stress, antioxidants, bone loss, postmenopausal women, polyphenols, micronutrients, osteoporosis, nutritional supplement

Abbreviations: TAC: Total antioxidant capacity; TEAC: Trolox-equivalent antioxidant capacity; ROS: Reactive oxygen species.

Introduction

Osteoporosis is a disease characterized by low bone mass and deterioration of the microarchitecture of bone tissue, leading to fragility fractures primarily in the hip, spine and wrist (1). Approximately one in three women and up to one in five men over the age of 50 will develop osteoporosis world-wide (2). Although several factors have now been identified as increasing the risk of osteoporosis, oxidative stress has now emerged as one of the most important life style risk factor associated with loss of bone mass (3-5).

Antioxidants capable of counteracting this effect have demonstrated to be important in decreasing the risk of osteoporosis (6-8). There has been an increased interest in the water-soluble antioxidant polyphenols as a result of in vitro and in vivo evidence demonstrating that they may protect against oxidative damage, mainly due to their antioxidative and free radical quenching properties, thus limiting the risk of various degenerative diseases associated with oxidative stress, including osteoporosis (9-13).

Nutritional supplements such as greens+ bone builder™ contain not only polyphenol rich plant constituents, but also other nutrients that were shown to be individually good for bone health. The composition of bone builder™ includes the following: vitamins B6, B12, C, D3, and folic acid; minerals such as calcium, magnesium, zinc, chromium, selenium, manganese,

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Received April 26, 2012
Accepted for publication June 4, 2012

boron, copper; and antioxidant lycopene. Previous in vitro results from our laboratory have shown that greens+™, a nutritional supplement rich in a variety of polyphenols is effective in stimulating osteoblasts to form bone nodules in a dose-dependent manner (14). Another nutritional supplement, bone builder™, which is rich in minerals, vitamins and nutrients, also had a significant dose-dependent stimulatory effect on bone nodule formation (15). When greens+™ and bone builder™ were tested as combination, the effects were six times more effective than either one alone (16).

This led us to believe that additive effects of greens+™ and bone builder™ may have a beneficial effect in reducing oxidative stress and reducing the risk of osteoporosis. The aim of this study, therefore, was to investigate the effects of the nutritional supplement commercially known as greens+ bone builder™, which combines the total polyphenols in greens+™ with various vitamins, minerals and antioxidants present in bone builder™, on biomarkers of oxidative stress in postmenopausal women who are at risk for osteoporosis.

Subjects and Methods

Participant Recruitment and Sample Collection

Participant recruitment was conducted from 2008-2011. Female participants between 50-60 years old, who were at least one year postmenopausal, were recruited using telephone and advertisements and asked to sign an informed consent in conformation to the guidelines of the St Michael's Hospital Research Ethics Board (REB). Exclusion criteria included participants who smoked cigarettes or were on medications affecting bone metabolism, including those for heart disease, high blood pressure, diabetes and/or osteoporosis. Based on these criterions, 48 subjects participated in the study. All participants were asked to provide a baseline 12-hour fasting blood sample at the first visit. Another sample of fasting blood was collected following a one-week washout period during which the no-green tea and herbal/vitamin supplements diet was maintained. The participants were then randomly assigned to either the greens+ bone builder™, N=24, (to be referred to as Treatment group) or the placebo, N=24, (to be referred to as Placebo group) for a period of 8 weeks during which they continued to refrain from consuming the restricted diet. Fasting blood samples were collected after 4 and 8 weeks on their respective treatments. Blood samples were processed to obtain serum.

Serum Analysis

Processing of Blood Samples

Unless otherwise specified, all materials were obtained from Sigma Aldrich Canada, Oakville, ON, Canada. Fasting blood samples collected from participants were centrifuged at 2,500 RPM within 1 hour of collection. The serum was then collected, aliquoted equally into 4 eppendorf tubes (minimum of 250 μl each) and keep frozen at -80oC. The frozen samples were thawed for analysis of total antioxidant capacity (TAC) (17), protein oxidation (thiols) (18) and lipid peroxidation (TBARS) (19).

Total Antioxidant Capacity

Total antioxidant capacity (TAC) was measured, using the Trolox-equivalent antioxidant capacity assay (TEAC) (17), to determine the overall capacity of antioxidants in vitro using 2,2, azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+), a blue-green chromophore. The absorbance was read at 734 nm to determine decolourization (Milton Roy Spectronic 1001 Plus, PA, USA), and compared to the decolourization produced by Trolox, a vitamin E analogue.

Oxidative Stress Parameters

Protein oxidation was determined by estimating protein-sulfhydryl groups (thiols) in serum (18). The optical density read at 412 nm (Milton Roy Spectronic 1001 Plus, PA, USA) and protein thiols were calculated using an absorptivity of 13600 cm-1 M-1. A high concentration of protein thiols corresponds to a lower protein oxidation.

Lipid peroxidation was measured in the serum using the thiobarbituric acid-malondialdehyde (TBA-MDA) assay. Briefly, the method as previously described is as follows (19): To an aliquot of serum sample were added the reagents BHT, orthophosphoric acid and TBA reagent and incubation carried out at 90ºC for 45 min followed by butanol extraction. The optical density of the pink-colored supernatant containing TBARS was read at 535 nm (Milton Roy Spectronic 1001 Plus, PA, USA) and reported as nmols of TBARS per ml of serum TBARS using an absorptivity of 156 mM-1cm-1 previously reported (19).

Statistics

All statistical analyses were performed using the latest version of SAS system (version 9.2; SAS Institute, Cary, NC, USA). Summary statistics of participant demographics such as age and BMI were generated and presented as means ± standard errors of the mean (SEM). Repeated-measures one-way ANOVA, with Tukey's multiple comparison test was used to test for significant differences in oxidative stress parameters and antioxidant status from the start of the treatment period to 4 and 8 weeks on the treatment. In cases where data were not normally distributed, the Friedman test with Dunn's

multiple comparison test was substituted. Percent change in oxidative stress parameters and antioxidant status were calculated using an unpaired t-test, or the Mann-Whitney test for data that were not normally distributed to analyze for significant differences between placebo and treatment groups. Significance was considered $p<0.05$.

Results

A total of 47 postmenopausal women, 24 in the Placebo group and 23 in the Treatment group (one participant withdrew due to personal reasons), completed the study. Table 1 describes the participant baseline characteristics. There were no significant differences among groups with respect to age, BMI, years since menopause, or blood pressure (Table 1). Similarly, there were no significant differences at baseline for average serum TAC (Treatment group, 1.45 ± 0.05 mM and Placebo group, 1.50 ± 0.05 mM) and protein oxidation (Treatment group, 469.7 ± 18.1 μM and Placebo group, 490.1 ± 12.5 μM). However, there was a significant difference between the Treatment group and Placebo group for TBARS at baseline with the Treatment group showing higher levels of TBARS at 7.1 ± 0.3 nmol/mL and 6.4 ± 0.2 nmol/mL, respectively (Table 1).

Table 1
Participant characteristics and baseline values for oxidative stress parameters and antioxidant capacity for each group

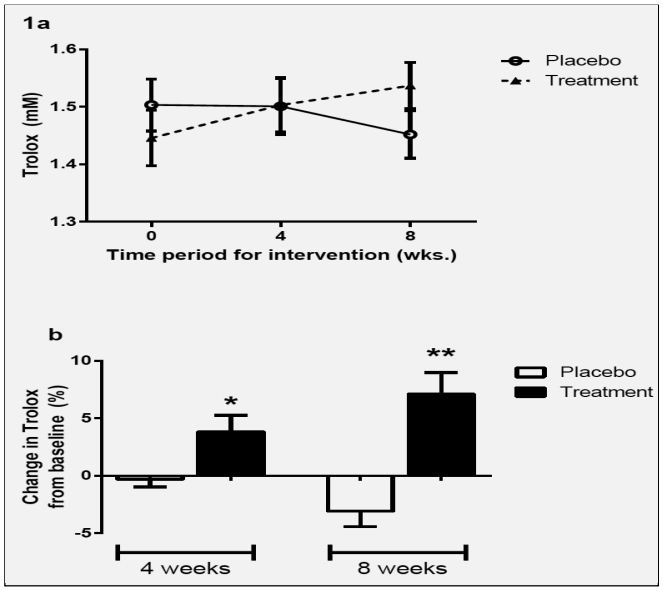
Parameters Measured	Placebo	greens+ bone builder™
Age (yrs)	55.5 ± 0.3	56.2 ± 0.6
Weight (lbs)	144.4 ± 5.1	143.7 ± 5.4
Height (inches)	63.7 ± 0.5	64.6 ± 0.6
Pulse Rate (beats/min)	67.0 ± 2.0	66.0 ± 2.0
Blood Pressure - systolic (mmHg)	115.6 ± 3.2	118.9 ± 3.2
Blood Pressure - diastolic (mmHg)	75.1 ± 2.0	72.5 ± 2.3
BMI (kg/m3)	25.0 ± 1.0	23.8 ± 0.8
Years since Menopause	5.7 ± 0.6	4.6 ± 0.5
Total Antioxidant Capacity (TEAC)(mM)	1.50 ± 0.05	1.45 ± 0.05
TBARS (nmol/mL)*	6.4 ± 0.2	7.1 ± 0.3
Protein Thiols (μM)	490.1 ± 12.5	469.7 ± 18.1

* Placebo values had significantly lower TBARS than the supplement group (unpaired t-test, $p<0.05$).

An interaction between time (4 and 8 weeks) and type of treatment (Treatment and Placebo group) was detected ($p<0.05$) for TAC such that TAC was greater in the Treatment group than in the Placebo group (Figure 1a) after treatment. Significant increases of 3.8 ± 1.4% and 7.1 ± 1.9% from baseline to 4 and 8 weeks among this group was also significantly different from the Placebo group ($p<0.01$, $p<0.0001$, Figure 1b).

There was an overall interaction between time (4 and 8 weeks) and type of treatment (Treatment and Placebo group) ($p<0.0001$) on lipid peroxidation, such that consuming the supplement rich in polyphenols and other micronutrients was lower than those who consumed the placebo (Figure 2a) after treatment. The Treatment group also resulted in a decrease of 6.6 ± 1.0% after 4 weeks, and to 10.0 ± 1.3% after 8 weeks in lipid peroxidation, which was significantly opposite to the 2.6 ± 1.7% and 4.3 ± 2.9% increase at 4 and 8 weeks of treatment seen among the Placebo group, respectively ($p<0.0001$, Figure 2b).

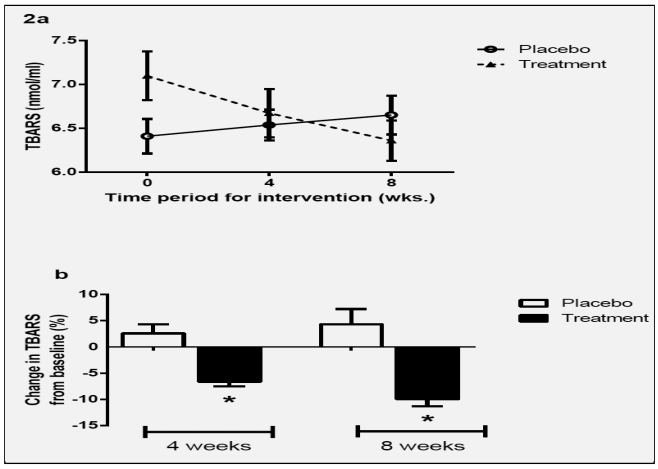
Figure 1
a) Total antioxidant capacity (TAC) in the serum over 8 weeks, as determined using the TEAC assay. Time by treatment effect values are expressed as mean ± SEM and were compared within supplement group using a repeated-measures ANOVA with Tukey's Multiple Comparison test ($p<0.05$) b) Change in total antioxidant capacity over the 8 week supplement period. The change in TAC was measured relative to baseline concentration. Values are mean % change ± SEM for each supplement group and were compared using an unpaired t-test (*$p<0.01$, **$p<0.0001$)



An interaction between time (4 and 8 weeks) and type of treatment (Treatment and Placebo group) was detected ($p<0.05$) on protein thiols, such that the Treatment group was higher in protein thiols, indicating a decrease in protein oxidation, compared to the Placebo group (Figure 3a) after treatment. There was a 3.2 ± 1.1% and 5.3 ± 1.1% increase in protein thiols from baseline to 4 weeks, and to 8 weeks among the Treatment group, which was significantly different from the decrease in protein thiols of 3.5 ± 2.9% after 4 weeks and by 2.5 ± 1.8% after 8 weeks seen within the Placebo group ($p<0.05$, $p<0.001$, Figure 3b).

Figure 2

a) Concentration of lipid peroxidation in the serum over 8 weeks, as determined by TBARS. Time by treatment effect values are expressed as mean ± SEM and were compared within supplement group using repeated-measures ANOVA with Tukey's Multiple Comparison test ($p<0.0001$). b) Change in lipid peroxidation over the 8 week supplement period. Change in lipid peroxidation was measured relative to baseline concentration. Values are mean % change ± SEM for each supplement group and were compared using an unpaired t-test (*$p<0.0001$)



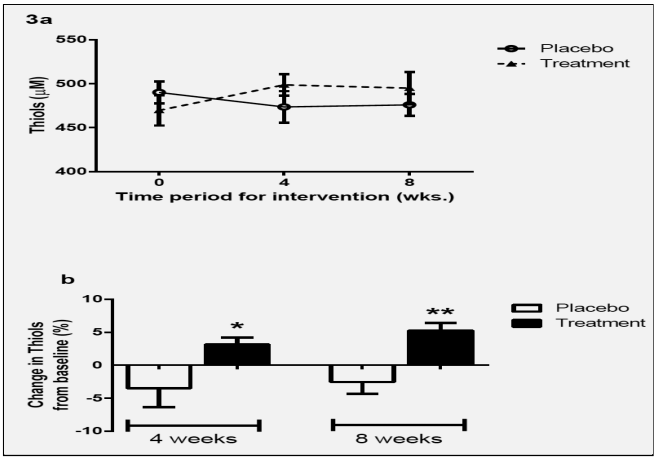
Discussion

Previous studies showing the antioxidant properties of polyphenols were based mainly on in vitro and animal studies. Our laboratory is the first to evaluate the efficacy of the nutritional supplement containing polyphenols and other micronutrients administered to postmenopausal women who are at risk for osteoporosis. The Treatment group showed increased TAC after 8 weeks of treatment. Furthermore, there was a significant change at 4 and 8 weeks between the Placebo and Treatment group. The TEAC method has been validated to measure the antioxidant properties of both the lipid- and the water-soluble antioxidants in vivo (20). This assay is used primarily for its ability to assess many endogenous antioxidants in addition to those found in the supplement and its capability to quench reactive oxygen species (ROS).

Although the polyphenols in greens+™ may have been the principle component of the nutritional supplement contributing to the observed antioxidant effect, the combined effect with other micronutrients and antioxidants in the bone builder™ may also have contributed to this effect (21, 22). Similar results obtained in our in vitro study showed that greens+ bone builder™ to be more effective than greens+™ or bone builder™ alone in stimulating bone nodule formation. Our study was not designed to study the interactive mechanisms between the various components of greens+™ and bone builder™. It is therefore, not possible to associate the antioxidant affects observed in the present study to any one component of green+ bone builder™.

Figure 3

a) Concentration of protein oxidation in the serum over 8 weeks as determined by thiols. Time by treatment effect values are expressed as mean ± SEM and were compared within supplement group using the Friedman's test with Dunn's Multiple Comparison test ($p<0.01$). b) Change in protein oxidation over the 8 week supplement period. Change in protein oxidation was measured relative to baseline concentration. Values are mean % change ± SEM for each supplement group and were compared using the Mann-Whitney test (*$p<0.05$, **$p<0.001$)



In addition to measuring TAC, other more sensitive biomarkers such as lipid peroxidation and protein oxidation to measure the effectiveness of the nutritional supplement in reducing oxidative stress. Greens+ bone builder™ significantly reduced oxidative stress compared to placebo groups after both 4 and 8 weeks. Previous in vitro, in vivo and clinical studies (9-12, 14) have also shown similar results where there was a decrease in oxidative stress parameters after the consumption of polyphenols. In a recent study, the effectiveness of lycopene, a fat-soluble antioxidant, on oxidative stress parameters in humans was investigated. The results showed decrease in oxidized proteins and lipid perioxidation at 4 weeks of treatment (23). This is similar to our finding where there was a significant decrease in both protein oxidation and lipid peroxidation within the treatment group when compared to placebo at 4 and 8 weeks of treatment.

A variety of dietary antioxidants has been shown to be capable of scavenging ROS directly. Previous studies demonstrated that a diet high intake of foods rich in polyphenols and other micronutrients, increasing the antioxidant capacity, has been linked to lowered risks of many chronic diseases involving oxidative stress (24-26).

As well, an in-depth review suggesting that the combination effect of carotenoids found in foods and supplements and may have a greater reduction in the risk of chronic diseases such as osteoporosis when compared to a single nutrient (27).

Since polyphenols are quickly metabolism in the human body, its concentration found in blood is low (<1 μmol/L) (28-30). This is such a low concentration to show any significant and direct antioxidant activities, thus some researchers believe that it is unlikely that polyphenols act as antioxidants in vivo (31-33). Thus, attention should be brought to the additive effect of micronutrients and polyphenols. It has also been noted that polyphenols may function as a co-antioxidant, and are involved in the regeneration of essential vitamins (32).

The relationship between the antioxidant property of the nutritional supplement and its effect on bone health was recently reported (14). They demonstrated that supplementation with greens+™ containing polyphenols such as quercetin, apigenin, kaempferol and luteolin was shown to increase the proliferation of human osteoblast-like cells at early time points of addition as well as to stimulate bone nodule formation in a dose- and time-dependent manner (14). These observations suggest a positive effect of ingesting polyphenol-rich foods and supplements in reducing oxidative stress, stimulating the activity of osteoblast cells, which may reduce the risk of osteoporosis.

In conclusion, our study has shown that daily supplementation of a combination of polyphenols and other trace nutrients and minerals in the form of the nutritional supplement, greens+ bone builder™, can significantly increase the antioxidant capacity and decrease the extent of oxidative damage in postmenopausal women at risk of osteoporosis. The beneficial effects can be due to the additive effect of the polyphenols with other vitamins and minerals found within the supplement. This observation along with previously observed bone-protective effects of the individual polyphenols, nutrients and trace minerals, has generated increased interest into further exploring them as a nutritional supplement that is beneficial to bone health. Although there is evidence that implicate oxidative stress is an important mediator of bone loss aiding in the development of osteoporosis, our laboratory is the first to investigate and show the combined effect of a variety of polyphenols alongside other micronutrients in healthy postmenopausal women, suggesting that this may be a good alternative for the prevention or treatment of osteoporosis.

Competing interests: The authors declare that they have no competing interests.

Acknowledgements: We would like to thank all the participants that took part in this study. We also thank Genuine Health Inc. for providing the supplement and placebo products used in this study. Authors acknowledge the financial contributions made by the Canadian Institute of Health research and Genuine Health, Toronto, Canada.

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