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Review Article

Effects of Vegetable Intake on Biomarkers Related to Oxidative Stress in Healthy Young Females

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Abstract

Recent studies show that vegetable consumption is effective in prevention of disease associated with oxidative stress including various cancers and cardiovascular disease. The aim of our study was to determine whether intake of vegetable for a certain period would affect biomarkers related to oxidative stress in young volunteers. Dietary interventions were performed by crown daisy (*Chrysanthemum coronarium L.*) trial (n=14) and broccoli (*Brassica oleracea L.*) and komatsuna (*Brassica campestris L.*) trials (both n=15). Subjects consumed specified vegetable (200g/day) for 7 days. Blood and urine samples were collected before and after trial and subjected to the estimation of reactive oxygen metabolites (ROM), urinary Isoprostane and biological antioxidant potential (BAP) levels. Following both of crown daisy and komatsuna trials, significant reduction of serum level of ROM was revealed ($p < 0.05$ and < 0.01, respectively). Significant reduction of urinary Isoprostane was observed after crown daisy and broccoli trials ($p < 0.01$ and < 0.05, respectively). And following both of crown daisy and broccoli intakes, significantly increased serum level of BAP was observed (both $p < 0.05$). However, contrary to our expectations, significant correlations among changes of such biomarkers were not confirmed and uptake of vegetables did not exhibit common effects on such serum and urine markers. It was indicated that dietary components in vegetables affected antioxidant efficacy and individual difference of baseline oxidative damage level influenced by various environmental factors also affected such efficacy. These findings suggested that modest antioxidant effects, contributing to prevention of disease, might be provided by these vegetables consumption.

KEY WORDS: antioxidant, BAP, Isoprostane, ROM, vegetable

Introduction

Oxidative stress is considered as one major risk factor in lifestyle-related disease including various cancers and cardiovascular disease [1,2]. Lifestyle habits such as alcohol intake, smoking and excessive exercise are also considered to have some relation to oxidative stress. It has been reported from epidemiological and clinical area that oxidative damage is involved in certain illness, lifestyle or other factors (*e.g.*, sex, age and body mass index) [3-5].

On the other hand, previous studies show that vegetable consumption is effective in prevention of disease associated with oxidative stress [6,7]. Since vegetables contain elements having antioxidant activity such as β-carotene, ascorbic acid, tocopherol and bioactive compounds of phytochemicals, a proactive intake of vegetables is recommended for general population in everyday life [8]. Dietary antioxidants function as an inhibitor of a radical chain reaction and as a scavenger of singlet oxygen promoting the reaction. Endogenous and exogenous antioxidants are essential to maintain defense system against oxidative damage to body tissue. It may be said that vegetable intake is high quality evidence as a preventive factor for oxidative stress related disease from the standpoint of primary prevention. It has been proven by the observation on consuming 12 servings vegetable and fruit (VF) for 14 days that diets rich in VF contribute to reduction in oxidative cellular damage markers [9]. However, the other published study in which 600 g VF/day was supplemented for 24 days has null findings on oxidative DNA damage [10]. Thus, there are various results from recent intervention studies on increased intake of vegetables. But these results do not demonstrate common findings. In addition, a decreasing effect of oxidative cell damage has been verified mainly at cruciferous family (*e.g.*, broccoli sprout) in vegetables [11]. As there are few evidences for the effect of vegetable intake on diet intervention trial, studies to determine such effect have been expected to be conducted.

The aim of this present study was [1] to investigate in healthy volunteers whether the regular uptake of vegetable for a certain period would provide beneficial effect on biomarkers concerning to oxidative stress and [2] to search a promising vegetable with preferable antioxidant performance. For the purpose of such investigation, diet intervention trial was conducted selecting certain kinds of vegetable with antioxidant activity confirmed among previous studies *in vitro* [12-14]. In order to determine overall state of oxidative stress *in vivo*, effect of supplementation with vegetable was evaluated in both aspects of 'pro-oxidant status' and 'antioxidant defense status' during the intervention trial. Following three biomarkers were preferred as markers representing pro-oxidant and antioxidant status because such biomarkers were considered to be useful as an indicator in biochemical screening for prevention of lifestyle related disease and in monitoring for dietary treatment process [4,15,16]. Pro-oxidant status was evaluated by measuring the level of serum reactive oxygen metabolites (ROM) reflecting the concentration of hydroperoxides and the level of urinary 8-isoprostaglandin $F_{2\alpha}$ (Isoprostane), which is the reliable index of overall lipid peroxidation. Antioxidant defense status was assessed by determining the biological antioxidant potential (BAP) in serum, which expresses the serum ferric reducing ability.

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Materials and Methods

Subjects and study design

This study constitutes of substudies #1 and #2 ('substudy #1' and 'substudy #2', respectively). The substudy #1 was conducted with 14 females (age, 21±1 yr; body mass index, 21.3 ± 2.9 kg/m^2). The substudy #2 was conducted with 15 females (age, 21±1 yr; body mass index, 20.5 ± 3.1 kg/m^2). All of these subjects were selected from the students of the Kagawa Nutrition University. For the purpose of this study, we excluded subjects who were smokers and took any kind of medicines or supplements. All subjects were instructed to maintain a healthy eating pattern, to avoid consuming alcohol and to not perform excessive exercise during the study period. This study was carried out in accordance with the Helsinki Declaration, and approved by the ethics committee of Kagawa Nutrition University and all participants gave informed consents.

The study design is adjusting gender, age and dietary and before-after study without control. Period of each trial in the substudies #1 and #2 was 14 straight days. For first 6 days, subjects could follow their habitual keeping common pattern of dietary intake and for next 7 days, they consumed specified vegetable (200 g/day) added to basal diet. Samples of blood and urine were collected at just before and after the treatments on the day 7 and the day 14, respectively.

The substudy #1 included a trial with a specified vegetable; crown daisy (*Chrysanthemum coronarium L.*). The substudy #2 included two trials with following specified vegetables; broccoli (*Brassica oleracea L.*) and komatsuna (*Brassica campestris L.*). An interval between broccoli and komatsuna trials was more than a month in which subjects had usual lifestyle pattern.

Dietary intervention

Added vegetables, which were considered to exhibit relatively high antioxidant activity by previous researchers [12-14], were available in Japan and suitable for blanching and freezing. The vegetables were purchased at a retail store and immediately processed. Only edible parts of the vegetables were blanched to inactivate oxidative enzyme including polyphenol oxidase. And after cooling at room temperature, blanched vegetables were divided into portions of 200 g flesh weight equivalent and frozen at –30°C. To avoid any other influence than added vegetables, the meals were prepared in a uniformed formulation and provided to the subjects. The basal diet of the substudies #1 and #2 provided energy and macro- and micro-nutrients based on the Dietary Reference Intakes for Japanese. Energy and nutrients contained in actually provided basal diet menus are shown in ***Table1***. During each trial, the subjects defrosted a defined amount of specified vegetable in a microwave oven for several minutes and consumed it with the basal diet at dinner time for 7 days.

Laboratory measurements

Pro-oxidant status was determined by derivatives of reactive oxygen metabolites test (ROM test, Diacron, Grosseto, Italy), spectrophotometric analysis, which measures serum concentration of hydroperoxides produced by reactive oxygen species and free radicals. The ROM test was performed on an analytical device of free radical analytical system 4 (FRAS4, Diacron, Grosseto, Italy). The total amount of hydroperoxides in serum is related to the reactive oxygen species and free radicals from which they are formed. Each sample of 20 µL blood was collected from a fingertip in the morning and analyzed promptly as follows. The blood sample (20 µL) was dissolved in acetate buffer solution, the resulting solution was decanted to a plastic cell containing chromogenic substrate, gently mixed, treated in a centrifuge (6000 rpm for 1 min at 37°C) and measured through spectrophotometric procedures (absorption at 505 nm). In this test, concentrations are expressed in conventional units (U. Carr) because various kinds of hydroperoxides in serum were analyzed. 1 U.Carr is equivalent to 0.08 mg/100 mL H_2O_2.

As antioxidant defense status, the serum ferric reducing ability was measured by performing biological antioxidant potential test (BAP test, Diacron, Grosseto, Italy), spectrophotometric analysis. The BAP test was also performed using the FRAS4 device according to the analytical procedure. Each sample of about 100 µL blood was obtained from a fingertip at the same time as ROM test and then a 10 µL serum sample was prepared from the whole blood by centrifugation (6000 rpm for 90 sec at 37°C) and measured immediately. The ferric chloride reagent of 50 µL was added a plastic cell containing the thiocyanate derivative reagent. The resulting solution was gently mixed and its absorbance was measured at 505 nm. Then, this colored solution was combined with 10 µL of the serum sample in the same cell, incubated for 5 min at 37°C, and its absorbance at 505 nm was determined. The measured values are expressed in concentration of ferrous ions (µmol) per liter of serum sample.

Urinary Isoprostane level was analyzed using competitive enzyme-linked immunosorbent assay kit from Oxford Biomedical Research Inc. (Oxford, MI, USA) according to the analytical procedure. The individual urine samples were prepared from total volume of collected 24 hr urine in plastic container. A part amount of such total urine was transformed into covered plastic tubes and stored at –30°C until performing the assay. As prior treatment, urine samples were thawed and diluted it five times with enhanced dilution buffer. The samples of 100 µL diluted urine samples were added to the 96-well microplate coated by polyclonal antibody and then 100 µL of the diluted 15-isoprostaneF2t HRP conjugate was added to each well and the resulting solution was incubated for 2 hr at room temperature. After washing with wash buffer for three times, 200 µL of TMB substrate was added to each well and the resulting

Table 1 Energy and macro- and micro-nutrients contained in provided basal diet menus.

Component	*substudy #1*	*substudy #2*
Energy (kcal)	1825 ± 102	1776 ± 70
Protein (%E)	18.4 ± 1.6	17.1 ± 1.0
Fat (%E)	23.5 ± 2.9	23.4 ± 3.3
Carbohydrate (%E)	58.2 ± 3.2	59.5 ± 2.6
Potassium (mg)	2622.4 ± 268.4	2540.5 ± 125.7
Calcium (mg)	535.0 ± 91.0	509.0 ± 77.5
Magnesium (mg)	295.0 ± 28.4	272.0 ± 26.6
Iron (mg)	7.8 ± 1.2	8.0 ± 1.5
Retinol equivalents (µg)	517 ± 90	512 ± 67
α-Tocopherol (mg)	9.2 ± 1.7	7.4 ± 1.0
Ascorbic acid (mg)	87 ± 17	70 ± 19
Total dietary fiber (g)	14.7 ± 1.8	14.6 ± 1.9
Salt equivalents (g)	7.6 ± 0.6	7.8 ± 1.0

All values are mean ± SD (standard deviation) per day.
Abbreviations: %E, percent of energy.
Nutrition analyses were performed by using the calculation software (Excel Eiyo-kun, Kenpakusha, Bunkyo-ku, Tokyo, Japan).

solution was incubated for 30 to 40 min. Then 50 µL of 3N sulfuric acid was added to each well to stop the reaction, and its absorbance was determined at 450 nm. The observed values were indicated by concentration of urinary Isoprostane per day (µg/day) that were calculated by multiplying conversion value from 15-isoprostaneF_{2t} standard by 24 hr urinary volume.

Determination of dietary antioxidant and radical scavenging activity

Antioxidants content and their radical scavenging activities were also assayed for the vegetables used on diet intervention trials (substudies #1 and #2).

The total polyphenolic content of the vegetables were estimated by the colorimetric method using Folin-Ciocalteu reagent, which we followed a procedure used in previous research [17]. Measurement vegetable samples were prepared by freezing dried and extracting with 80% (v/v) ethanol solution for 30 min. Chlorogenic acid was used as a standard substance to measure. Polyphenol was provided as an amount of total polyphenol, because the measurement method employed in this study quantified several polyphenoles including flavonoids and anthocyanin.

The amount of ascorbic acid in the vegetables were measured using high-performance liquid chromatography (HPLC) analysis according to Explanation on Analytic Manual of Food Recorded in 5th Revised Standard Table of Food Composition in Japan [18]. The vegetable samples were thawed, shred finely, extracted by grinding with 5% (w/v) metaphosphoric acid and diluted with 5% metaphosphoric acid. The column (Finepak SIL-5, size: 250 × 4.6 mm, JASCO Co., Hachioji-city, Tokyo, Japan) was used at 40°C at a flow rate of 1.5 mL/min and an injection volume of 20 µL.

β-carotene was also measured using HPLC analysis according to the above analytical manual [18]. Just as measurement of ascorbic acid, so vegetable samples were thawed, shred finely, extracted by grinding with ethanol and diluted with ethanol. The column (Mightysil RP-18 (L) GP, size: 150 × 4.6 mm, Kanto Chemical Co., Inc., Chuo-ku, Tokyo, Japan) was used at 40°C at a flow rate of 1.5 mL/min and an injection volume of 20 µL.

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was measured by the method of Takahashi and Higuchi [19]. The vegetable samples were prepared by freezing dried and extracting with 80% (v/v) ethanol solution for 2 hr in a similar manner to the total polyphenol estimation. In this study, BHA (butylated hydroxyanisole) was used as a standard substance.

The values of antioxidants of the above vegetables and their radical scavenging activities were expressed in a daily portion of 200 g flesh weight equivalent of each vegetable (***Table 2***).

Statistical analysis

Biological data were presented as median and quartile deviation (QD) unless stated otherwise. As data of biomarkers did not show normal distribution, the median of the data were compared with a non-parametric test, Wilcoxon signed rank test between before and after trial. Additionally, because it was estimated that the biomarkers might decrease oxidative damage and increase antioxidant ability due to uptake of vegetable, the correlative analysis was performed with the Spearman rank correlation coefficient between the biomarkers. The differences were considered significant at $p < 0.05$ and all analyses were performed using the SPSS 19.0J for Windows (IBM Japan, Ltd., Chuo-ku, Tokyo, Japan).

Results

Effects of vegetable consumption on serum and urine biomarkers are shown in ***Table 3***. Serum ROM level decreased statistically significantly following crown daisy trial (–4.3%; $p = 0.011$) and uptake of komatsuna also revealed statistically significant decrease of serum ROM level (–5.6%; $p < 0.01$). Uptakes of crown daisy and broccoli raised serum BAP level in a statistical significance (+2.0%; $p < 0.05$ and +5.8%; $p = 0.013$, respectively). Uptake of crown daisy was found to reduce urinary Isoprostane in a statistical significance (–13.7%, $p = 0.006$). After broccoli trial, urinary Isoprostane was reduced in statistical significances (–14.5%; $p = 0.013$), while after komatsuna trial Isoprostane level did not decrease significantly.

Table 4 shows the results of correlation analysis of the changes in the levels of the biomarkers. It is considered that improvement of the oxidative stress balance will result in a negative correlation between ROM and BAP levels. However, the correlation coefficients obtained from the present study exhibited positive values. If oxidative damage is reduced by uptake of vegetables, a positive correlation is expected between ROM level and urinary concentration of Isoprostane. Such tendency was observed only for uptake of broccoli though not significant. It is also expected that there might be the negative correlation between BAP and Isoprostane levels by uptake of vegetables in a similar manner to the correlation between ROM and BAP levels. In this study, the negative correlation was found for uptakes of crown daisy and komatsuna though not significant.

Table 2 ***Content and radical scavenging activity in a daily portion of 200 g flesh weight equivalent of each vegetable on substudies #1 and #2.***

Sample	Total polyphenol (mg CA eq.)	Ascorbic acid (mg)	β-carotene (µg)	DPPH radical scavenging activity (µmol BHA eq.)
Substudy #1				
Crown daisy	381.4 ± 4.7	5.6 ± 0.8	9660 ± 206	2443 ± 226
Substudy #2				
Broccoli	203.6 ± 16.1	49.5 ± 10.1	1575 ± 47	763 ± 33
Komatsuna	53.5 ± 0.7	21.3 ± 2.9	5403 ± 86	717 ± 44

All values are mean ± SD per 200 g flesh weight equivalent.
Abbreviations: CA, chlorogenic acid; BHA, butylated hydroxyanisole, eq; equivalent.

Table 3 Changes in serum and urinary oxidative damage and antioxidant biomarkers in substudies #1 (n=14) and #2 (n=15).

Biomarkers and trial group	Before	After	% Change [1]	*P* value [2]
ROM (U.Carr)				
Substudy #1				
Crown daisy	309.8 ± 32.1	288.0 ± 36.6	− 4.3 ± 3.9	0.02
Substudy #2				
Broccoli	307.0 ± 30.0	306.0 ± 35.3	− 0.2 ± 4.4	ns
Komatsuna	309.0 ± 18.5	290.0 ± 22.3	− 5.6 ± 4.6	0.01
BAP (μmol/L)				
Substudy #1				
Crown daisy	2063 ± 120	2127 ± 113	2.0 ± 3.6	0.05
Substudy #2				
Broccoli	1986 ± 142	2077 ± 120	5.8 ± 4.1	0.02
Komatsuna	2021 ± 102	2012 ± 73	− 2.0 ± 4.3	ns
Isop (μg/day)				
Substudy #1				
Crown daisy	1.13 ± 0.39	0.83 ± 0.30	− 13.7 ± 11.6	0.01
Substudy #2				
Broccoli	0.96 ± 0.54	0.77 ± 0.27	− 14.5 ± 23.4	0.02
Komatsuna	1.15 ± 0.43	0.89 ± 0.19	− 9.0 ± 27.4	ns

Values are median ± QD (quartile deviation). Abbreviations: ROM, reactive oxygen metabolites; BAP, biological antioxidant potential; Isop, 8-isoprostaglandin $F_{2\alpha}$; ns, not significant.
[1] % Change was calculated according to the following equation: % Change = (after / before) × 100 − 100.
[2] Difference between before and after trial by Wilcoxon signed rank test.

Table 4 Correlations among changes of serum and urinary biomarkers by uptake of vegetables.

Trial group	ROM vs. BAP		ROM vs. Isop		BAP vs. Isop	
	r [1]	*p* [2]	*r*	*p*	*r*	*p*
Substudy #1						
Crown daisy	0.13	0.667	-0.25	0.383	-0.29	0.311
Substudy #2						
Broccoli	0.06	0.830	0.17	0.541	0.33	0.237
Komatsuna	0.40	0.140	-0.14	0.612	-0.48	0.069

n = 14 in substudy #1, n = 15 in substudy #2.
Abbreviations: ROM, reactive oxygen metabolites; BAP, biological antioxidant potential; Isop, 8-isoprostaglandin $F_{2\alpha}$.
[1,2] Correlation coefficients and significance levels among percentage changes of biomarkers at each trial by Spearman rank correlation coefficient.

Discussion

Our results suggest that uptake of crown daisy, broccoli and komatsuna suppress oxidative damage of serum and urine and/or improve serum antioxidant ability.

Crown daisy consumption

The results of influence on biomarkers related to oxidative stress by crown daisy consumption were explained on the basis of the following factors. Because crown daisy is rich in β-carotene exhibiting high lipid peroxidation inhibitory activity [20], it was suggested that crown daisy treatment might lead to improvement of oxidative damage, decreasing serum ROM level and reducing urinary Isoprostane level. Serum ROM level reflects concentration of hydroperoxides generated by oxidation of biomolecules with reactive oxygen species and free radicals. And urinary Isoprostane is produced by free radical catalyzed peroxidation of membrane and LDL phospholipids. Then it was suggested that crown daisy treatment might relate to polyphenol compounds, a kind of phytochemicals which were expected to have antioxidant activity. It was shown that crown daisy was rich in polyphenol and had a particularly high radical scavenging ability as listed on ***Table 2***. Polyphenoles in crown daisy, which are mainly phenylpropanoid such as chlorogenic acid (5-caffeoyl quinic acid) and isochlorogenic acid, are considered to contribute to radical scavenging activity, including their degradation product such as caffeic acid [12,21,22]. As the effect of chlorogenic acid on suppressing oxidative damage has been indicated by research of Kasai *et al.* [23] using rats, it is suggested there is possible beneficial effect of crown daisy consumption *in vivo*.

Broccoli consumption

Following factors were considered regarding the results of biomarker changes observed during broccoli trial. In recent similar study, Riso *et al.* [24] found a significant decrease in the level of enzyme introduced to repair oxidative damage after broccoli treatment in young males for 10 days, which provided the almost same amount of β-carotene and ascorbic acid as the present study. It was shown that urinary Isoprostane level as oxidative damage marker decreased in broccoli trial conducted in the present study and antioxidant effect of broccoli uptake on healthy subjects was suggested as the above research reported. Furthermore ascorbic acid in broccoli was considered to be a factor to change of serum BAP level. BAP level was measured by the color fading reactions where the ferric ions were reduced to ferrous ions from ferric ions using ferric chloride containing reagent. Recently it was reported that increase of BAP level depended on the concentration of ascorbic acid *in vitro* [25]. It was considered that such elevated level of BAP might be attributed to high reducing power of ascorbic acid. Although absorption of ascorbic acid into body was not examined in our trial, elevated BAP level was thought to be caused by increased supply of ascorbic acid during broccoli trial.

Komatsuna consumption

It was observed that serum ROM level decreased following komatsuna trial. Komatsuna sample used in this study moderately contained ascorbic acid and exhibited a relatively high free radical scavenging activity as shown in ***Table 2*** just like a previous research *in vitro* [14]. It has been shown the activity of nitric oxide synthase (iNOS) introducing generation of nitric oxide which is a kind of free radicals in mouse cell line was markedly inhibited by addition of the extract from komatsuna [26]. However, dietary intervention trial by komatsuna consumption has not been conducted *in vivo* until now.

Another involvement of functional food factor in cruciferous vegetables belonging to komatsuna and broccoli was considered. Cruciferous vegetables are characterized by containing isothiocyanates which may have effects in preventing cancer from several reports [27,28]. Especially broccoli contains high level of sulforaphane that is a kind of the isothiocyanates and may provide protective effect against oxidative damage from reactive oxygen species. Although the isothiocyanates in vegetable sample was not examined in the present study, it was considered that there was possibility of positive changes of biomarkers related to oxidative stress due to uptake of broccoli and komatsuna of cruciferous family because these phytochemicals had no small effect on biological oxidative damage status as shown in previous studies. Additionally, crown daisy and komatsuna have been edible only in Japan and/or East Asia, few intervention studies *in vivo* have been carried out actually. Further studies about vegetables with highly antioxidative effect including these plants are required.

Correlations among levels of biomarkers

In ***Table 4***, since expected correlations among levels of biomarkers were not confirmed in this study, uptake of vegetables for 7 days did not show uniform effects on serum and urine markers. This might be caused by a shortage of intervention period, because serum biomarkers generally, reflect short-term effects compared with urinary ones [3]. On the other hand in a precedent research to reduce oxidative stress by increasing vegetable intakes for healthy subjects, it has been reported that the range of subjects' baseline level of urinary Isoprostane is wide and changes of the marker after the intervention depend on lipid peroxidation status of the individual at baseline time before the one [29]. And decreased rate of urinary Isoprostane was reported be greater in the subject with higher level of Isoprostane at baseline than in the subject with lower level of one. Thus it was suggested that the subjects with such higher level of Isoprostane might have greater benefit from vegetable supplementation. Our results also indicated a similar tendency with the previous finding (though data were not shown). Although the basis for individual difference of baseline of urinary Isoprostane had not been identified in the previous study, it was mentioned that lipid peroxidation status could be influenced by change of lifestyle practices as eating pattern. To investigate such individual difference, it is considered that analysis after separating subjects based on oxidative damage levels and baseline survey of factors related to oxidative stress (*e.g.*, food intake and physical activity) before the intervention might be effective.

We have not set the control group because we had an idea to connect this study to further advanced study focused on an individual vegetable used in this study if possible. We think that setting the period for consumption of only basal diet or the control group for such consumption may give us more reliable results. Thus, our findings might provide some knowledge that would lead to improvements in oxidative stress by the uptake of vegetables.

Conclusion

The present study demonstrated that crown daisy, broccoli and komatsuna consumption can be effective in decreasing oxidative damage and/or increasing antioxidant ability in healthy young females. And then it was indicated that dietary components such as β-carotene and ascorbic acid in vegetables affected antioxidant efficacy and individual difference of baseline oxidative damage level influenced by various environmental factors also affected such efficacy. These findings suggest that modest antioxidant effects contributing to prevention of disease might be provided by the uptake of these vegetables, and at the same time these results must be interpreted in considering oxidative stress status in the individuals and number of factors affecting lifestyle and living environmental. From now on, further studies regarding dietary phytochemicals in vegetables and their absorption are necessary in order to elucidate the potential role of vegetables on the antioxidant benefit.

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Conflict of interest statement

The authors declare no financial or other conflicts of interest in the writing of this paper.

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