

Original Research

Supplementation with Mixed Fruit and Vegetable Juice Concentrates Increased Serum Antioxidants and Folate in Healthy Adults

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Objective: Epidemiological studies have shown that low plasma levels of antioxidant micronutrients, which are commonly found in fruit and vegetables, are associated with increased risk for diseases such as heart disease, cancer, metabolic disorders and the like. The aim of this study was to monitor the dietary habits of a group of healthy, middle-aged, men and women and to assess the effect of supplementation with a natural phytonutrient preparation from fruits and vegetables, on plasma levels of various antioxidant micronutrients and oxidative stress assessed by measuring 8-oxodGuo (8-oxo-7,8-dihydro-2'-deoxyguanosine) in urine.

Methods: The study followed a double-blind randomized cross-over design involving 59 healthy men and women (40–60 years of age). The supplement or a placebo was given to two groups for a total period of 14 weeks (crossover week 7). Blood levels of β -carotene, vitamin C, vitamin E, selenium and folate were measured at 0, 7 and 14 weeks. Fruit and vegetable consumption was monitored by means of a retrospective food frequency questionnaire at week 0, 7 and 14. Urinary 8-oxodGuo was also determined at these time points.

Results: Significant increases in blood nutrient levels after active supplementation were observed for β -carotene, vitamin C, vitamin E, selenium and folate. Ranges measured, after supplementation, often fell into those associated with a reduced risk for disease. Our data suggests that, although generally health conscious, participants still fell short of the recommended five portions of fruit and vegetables per day. No significant group changes were noted for 8-oxodGuo concentration in urine.

Conclusion: Supplementation with mixed fruit and vegetable juice concentrates effectively increased plasma levels of important antioxidant nutrients and folate.

INTRODUCTION

Epidemiological studies have shown the importance of a diet rich in fruit and vegetables in the prevention of illnesses, such as, heart disease, cancer, metabolic disorders and the like [1–4]. Low plasma levels of micronutrients with antioxidant properties, often found in fruit and vegetables, are associated with increased risk for these diseases [5–7].

Recommendations for consumption of fruit and vegetables are currently given as five or more servings per day [8,9]. In reality, however, surveys have shown that these guidelines are

rarely met [10,11]. In an effort to attain improved plasma levels of antioxidants, micronutrient supplementation has been suggested as a viable approach [12,13].

Previous intervention studies involving micronutrient supplementation have not always achieved the desired results [14,15]. It was reported that β -carotene-supplemented smokers were found to have a slightly increased lung cancer mortality when compared to the group not receiving the β -carotene supplement. The reason for this could be imbalanced administration of supplements. High doses of a single, synthetically produced, nutrient fail to consider the potential interactions and

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synergistic effects of these bioprotective nutrients [16]. Krinsky pointed out that β -carotene is very susceptible to oxidative destruction such as that occurring during times of smoking [17]. Further, the protection of β -carotene against oxidative damage is dependent on the presence of numerous other antioxidant factors [18]. There are many references to the important interactions of secondary plant-based substances which potentially act together with natural vitamins to produce a protective effect [19].

In contrast to other intervention studies which have looked at supplementation of high risk groups, the aim of this study was to monitor the dietary habits of a group of healthy, middle-aged men and women and to assess the effect of supplementation with a natural phytonutrient and antioxidant preparation, derived from a mixture of dried fruit and vegetable juices, on plasma levels of various antioxidant micronutrients.

MATERIALS AND METHODS

Subjects

Fifty-nine healthy Austrian men and women (between 40 and 60 years of age) were recruited by means of a newspaper advertisement. Table 1 shows the subject characteristics. Potential study subjects were informed, via formal presentation and individual interviews, about the study content and design. Written, informed consent was obtained from all study participants prior to entering the trial.

Subjects underwent a clinical examination to determine suitability for participation in the study. Exclusion criteria were severe or chronic illness; history of gastro-intestinal surgery or chronic bowel disease; current metabolic illness, such as diabetes mellitus, untreated hyper- or hypothyroidism or severe lipid metabolism disorder; regular intake of nutritional supplements (i.e. vitamin/mineral supplements or micronutrient-enriched foods), unless discontinued for at least two weeks before study entry and throughout the investigation; current pregnancy or breast feeding.

Study Design

The study followed a double-blind randomized cross-over design. Participants were asked to continue with their usual

diets. Subjects were instructed to refrain from taking any supplements or fortified foods or drinks for two weeks before the study began and for the duration of the study. To monitor fruit and vegetable consumption subjects completed retrospective food frequency questionnaires at the start of the study, week 7 and week 14. The subjects were divided randomly into two comparable groups (Table 1). Either supplement or placebo was given for a total trial period of 14 weeks with crossover at week 7.

Blood Sample Treatment

Blood samples were taken from all subjects at baseline, week 7 and week 14, and serum levels of the micronutrients β -carotene, vitamin E, folate and plasma levels of vitamin C were measured. Venous blood samples for the measurement of vitamin C were collected into lithium-heparinized polystyrene tubes and protected from light. After centrifugation the plasma was stored at -20°C and measured after collection within two days. Venous blood samples for the determination of α -tocopherol, β -carotene, selenium and folate were obtained using tubes without additives and centrifuged after coagulation. Serum samples were stored at $2-8^{\circ}\text{C}$ (folate and selenium) or -20°C (vitamin E and β -carotene) and measured after collection within two days.

Vitamin C was measured by reverse-phase HPLC and photometric detection. β -carotene and α -tocopherol were determined using HPLC after liquid/liquid extraction and detected with a UV/Vis detector (Shimadzu SPD-10AV). Folate was measured in an electro-chemi-luminescence-immuno assay (ECLIA) using an Elecsys 2010 system (Roche Diagnostics, Mannheim, Germany). Selenium was measured by electrothermal atomic absorption spectroscopy (Varian SpectraAA-400 Zeeman).

Urinary Sample Treatment

The urinary measurement of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) is an indicator of oxidative modification of cellular DNA [20,21], not routinely used in clinical practice. To measure 8-oxodGuo, first morning urine samples were collected, centrifuged at 2000g for 10 minutes, then samples of the supernatant were stored at -20°C until measurement within two days. All samples were measured at the same time. For the determination of 8-oxodGuo a competitive *in vitro* enzyme-linked immunosorbent assay from JAICA, Harouka, Japan was used. The sample and 8-oxodGuo monoclonal antibodies were added to the 8-oxodGuo precoated plates. The color reaction after different washing steps with the chromatic substance 3,3',5,5'-tetramethylbenzidine indicated the amount of antibody bound to the plate. Results were calculated as nmol/mmol creatinine.

Table 1. Subject characteristics by study group

Continuous Characteristics Mean \pm Standard Deviation	Group 1 (n = 30)	Group 2 (n = 29)	p-value
Age (years)	49.2 \pm 6.2	50.2 \pm 5.7	0.53
Weight (kg)	71.9 \pm 14.1	74.3 \pm 17.4	0.57
Height (cm)	170.7 \pm 10.4	169.6 \pm 9.0	0.64
Body Mass Index (kg/m ²)	24.8 \pm 5.2	25.8 \pm 5.8	0.47
Discrete Characteristics	Group 1	Group 2	
Number of Male Subjects	14	12	
Number of Female Subjects	16	17	
Number of Smokers	5	6	

Nutrient Supplement

The phytonutrient and placebo (calcium carbonate) supplements were provided in the form of opaque gelatine capsules (Juice Plus+®, NSA International, Memphis, TN). For the fruit capsules, apple, orange, pineapple, papaya, cranberry and peach were juiced and concentrated to powder using a proprietary low temperature drying process. For the vegetable capsules the same process was performed on juice from carrot, parsley, beet, broccoli, kale, cabbage, spinach, and tomato. Many of these fruits and vegetables are rich sources of β -carotene and other carotenoids as well as various vitamins, folates and minerals. The phytonutrient concentrate capsules also included B vitamins and folate, *Dunaliella salina*, acerola cherry and soy-derived d- α -tocopherol to provide standardized concentrations of natural β -carotene, calcium ascorbate and α -tocopherol, respectively (Table 2). The fruit capsules contained 850 mg fruit juice concentrate powder each. Two per day were taken with the morning meal. The vegetable capsules contained 750 mg each of vegetable juice concentrate powder. Two per day were taken at lunch or with a meal later in the day. The dosing schedule for the placebo was identical. All subjects took the phytonutrient supplements for 49 days and the placebo supplements for 49 days in a randomly assigned order.

Statistical Analysis

Numerical data were analyzed using SPSS 10.0 for Windows. Results were expressed as mean and SD. Statistical comparisons were made using Student's *t* test for paired or unpaired data as well as one-way ANOVA, as appropriate. *p*-values <0.05 were considered as statistically significant.

RESULTS

Subjects and Compliance

Of the 59 participants entering the study, 55 completed, with two subjects dropping out from each group. Of these four

Table 2. Composition of the phytonutrient supplement

	Fruit Mixture (values for 2 capsules)	Vegetable Mixture (values for 2 capsules)
Vitamin E	30 mg	30 mg
Vitamin C	150 mg	50 mg
Thiamin	0.4 mg	0.6 mg
Riboflavin	0.3 mg	1.0 mg
Niacin	7 mg	13 mg
Vitamin B6	2.0 mg	1.5 mg
Folate	100 μ g	300 μ g
β -carotene	6 mg	9 mg
Manganese	0.3 mg	1.5 mg
Chromium	18 μ g	30 μ g
Zinc	2.0 mg	2.5 mg
Magnesium		45 mg
Selenium		35 μ g

subjects, three did not attend all the laboratory appointments, and one consumed a nutrient enriched product. All test supplements were well tolerated. All capsules were reported, by questionnaire, to have been taken as directed. In addition, subjects were asked to return pill containers to allow compliance to be calculated based on returned pill count.

No significant differences between mean fruit and vegetable intake at baseline were determined between the two groups. About 70% of each study group recorded daily fruit intake (group 1 mean fruit intake 2.2 portions/day, group 2 mean fruit intake 2.4 portions/day) and about 30% recorded daily vegetable intake (group 1 mean vegetable intake 2.9 portions/day, group 2 mean vegetable intake 3.5 portions/day). No significant differences in fruit and vegetable intake were determined between the two groups at week 7 and 14. The only exception was the intake of cooked vegetables, which was significantly higher in group 2 at week 7; however, the intake of raw vegetables was significantly lower for this group during this period. Regardless of the groups' mean intakes, none of the individuals in either group achieved a mean of five or more portions of fruit/vegetables per day (results not shown).

Serum Micronutrients

The average serum micronutrient concentrations for group 1 and group 2 are presented in Table 3. Comparing placebo with active treatment, significant increases were recorded at week 7 for selenium ($p < 0.001$), β -carotene ($p < 0.001$), vitamin C ($p < 0.001$), vitamin E ($p < 0.01$) and folate ($p < 0.001$). At week 14, selenium ($p < 0.001$), β -carotene ($p < 0.001$), vitamin E ($p < 0.05$) and folate ($p < 0.001$) concentrations were significantly higher in the active group.

While the vitamin C status in both groups at baseline was found to be significantly different ($p < 0.05$), plasma vitamin C in group 2 still rose to similar concentrations achieved by group 1 during their active supplementation period.

Urine 8-oxodGuo

No significant differences between groups were recorded for urinary 8-oxodGuo levels during the study. However, there were disparate findings with regard to the observed declines. Group 1 showed a non-significant decline after consuming the active supplement 7 weeks while group 2, on placebo for this period, remained unchanged. Both groups had significant ($p < 0.001$) declines from baseline at the end of 14 weeks (Table 4).

DISCUSSION

These results show a positive effect of supplementation with enriched dehydrated fruit and vegetable juice powder on blood levels of β -carotene, vitamin C, vitamin E, selenium and folate. As has been shown previously, enhanced antioxidant status can help reduce deleterious oxidative processes [22,23]. Use of a

Table 3. Plasma levels of micronutrients before and after supplementation with a phytonutrient supplement or a placebo

	Group 1 ^(a)						Group 2 ^(b)					
	Baseline		Week 7		Week 14		Baseline		Week 7		Week 14	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Beta carotene (µg/L)	707	547.7	2495***	1648.5	731***	484.3	562	550.2	390***	315.7	2476***	1103.8
Vitamin C (µg/mL)	4.1*	1.9	8.8***	2.9	7.5	2.6	3.1*	1.3	6.1***	2.8	8.3	2.9
Vitamin E (mg/L)	14.1	3.5	19.7**	4.2	16.9*	4.3	14.8	3.4	16.9**	3.5	19.6*	5.5
Folate (ng/mL)	8.8	3.5	16.2***	5.8	9.0***	2.9	8.7	3.1	7.6***	2.9	15.0***	4.6
Selenium (µg/L)	91.9	20.6	110.2***	17.3	85.4***	9.8	93.0	23.0	90.5***	19.2	95.5***	12.1

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ = significant differences between groups.

(a) Group 1 (Supplement – Baseline to 7 weeks/Placebo – 7 to 14 weeks).

(b) Group 2 (Placebo – Baseline to 7 weeks/Supplement – 7 to 14 weeks).

Table 4. Measurement of 8-oxo-7,8-dihydro-2'-deoxyguanosine (nmol/mmol creatinine) (8 oxodGuo)

Group	Baseline		Week 7		Week 14	
	mean	SD	mean	SD	mean	SD
1	5.6	2.7	4.9	2.5	2.8	0.8
2	4.6	1.8	4.8	2.7	2.3	1.1

well balanced supplement concentrated from mixed fruit and vegetable juices could, therefore, be beneficial.

Mean baseline β-carotene levels were found to be higher (group 1, 707 µg/L, group 2, 562 µg/L) than reported in American subjects (mean 70 µg/L) [22], and in Finnish subjects (median 170 µg/L) [14], but comparable with French subjects in a study on the effects of vitamin and mineral supplementation on institutionalized elderly patients (471.14 µg/L) [24]. These differences may be due to the relatively healthy diets consumed by the trial subjects of our study.

Serum levels of β-carotene rose significantly and comparably in week 7 for group 1 (2495 µg/L) and week 14 for group 2 (2476 µg/L). A possible explanation for this substantial increase may be the high bioavailability of β-carotene in the supplement [22,23]. Furthermore, the action of β-carotene in the presence of other food factors, such as non-nutritive phytochemicals, could play a role. It has been proposed that the additive and synergistic effects of phytochemicals in fruit and vegetables are responsible for their antioxidant activity [25]. A supplement derived from dehydrated mixed fruit and vegetable juices could mimic the antioxidant behavior of fruits and vegetables.

There has been much discussion regarding β-carotene and risk of lung cancer in smokers following the Alpha Tocopherol Beta-Carotene (ATBC) study [14] and the Beta Carotene Retinol (CARET) study [15]. However the Chinese Cancer Prevention Trial [26] found a significant beneficial effect on mortality due to cancer when supplementing with a combination of β-carotene (15 mg), vitamin E and selenium. Wheatly clearly pointed out [16] that the effect of isolated β-carotene in high dosages cannot be compared to β-carotene in the presence

of other antioxidant nutrients. Both the ATBC and the CARET studies used high dose synthetic forms of single nutrients, which do not take into account the potential synergistic effects of micronutrients in combination. In view of the various research findings, supplementation with high doses of β-carotene only should be approached with caution, particularly in current smokers [27].

In our study mean baseline concentration of vitamin C was below threshold plasma levels of 8.8 µg/mL equated with a minimal risk for cancer and CVD [28,29]. These values appear lower than expected considering the relatively high proportion of fruit and vegetables consumed by the participants; however, Galan *et al.* [24] also reported baseline vitamin C levels of 3.4 µg/mL in elderly French patients. After seven weeks of supplementation with the active product, both groups achieved significant increases in plasma vitamin C levels reaching comparable values of 8.8 µg/mL (group 1) and 8.3 µg/mL (group 2).

Baseline serum levels of vitamin E were found to be higher than the threshold value of 12.9 mg/L associated with minimal risk of CVD and cancer in previous epidemiologic studies [28,29]. However, in a study investigating immune function in elderly smokers and non-smokers during supplementation with fruit and vegetable juice concentrates similar to the active tested in this investigation [30], baseline serum levels were also higher at 22.56 mg/L. This study reported significantly improved immune function after supplementation along with increased serum concentration of α-tocopherol to 28.75 mg/L. This suggests that further benefit may still be gained from vitamin E status greater than 12.9 mg/L, when the additional vitamin E is provided in a matrix of fruit and vegetable juice powder concentrates.

Although the recent GISSI-Prevenzione trial [31] of subjects with established heart disease and the MRC/BHF Heart Protection Study [32] discovered no significant benefits from vitamin E supplementation, consideration should be made for the fact that in these studies vitamin E was provided in high doses either isolated or in combination with only two other micronutrients (vitamin C and β-carotene).

Although the amount of selenium provided in the active supplement was relatively small (35 µg) a significant increase

in serum selenium was reported for group 1 subjects. This may be due to the bioavailability of the supplement [23]. Baseline selenium levels were found to be above the mean selenium concentrations reported for Austria of approximately 67.5 $\mu\text{g/L}$ [33] and may be a reflection of the healthy diets consumed by our study group. It has, however, been shown that in some parts of Europe soils are a poorer source of selenium than in the United States [33,34]. These considerations suggest that raising the plasma selenium levels of Europeans, by supplementation or a diet richer in selenium, could be beneficial.

Folate, though not specifically classified as an antioxidant, is an essential nutrient necessary for cell development [35]. In particular, methyltetrahydrofolate acts as a donor of one-carbon groups required for methylation reactions, such as the synthesis of methionine from homocysteine [35]. Increased concentrations of homocysteine are a risk factor for occlusive vascular disease [36]. Trials have been carried out demonstrating reduction of serum homocysteine through supplementation with folic acid [37–39]. Baseline serum levels of folate (Table 3) are comparable with those reported at baseline by Wald *et al.* [37] of 8.12 ng/mL. In our study, 0.4 mg folate contained in the active supplements achieved significant increases in serum folate levels for both groups during the supplementation period. Although homocysteine was not determined in our study, two comparable studies did establish a reduction in homocysteine following intervention with a mixed fruit and vegetable juice concentrate [40,41].

The results of the urinary measurements of 8-oxoGuo (Table 4) are not easily reconciled with the supplementation scheme. Values at the end of the study are clearly lower, which could be interpreted as an effect of the intervention; however, there are no significant differences between the two groups at the time of the crossover. Various theories on the elimination of urinary 8-oxoGuo through antioxidant supplementation have been published. Some authors have reported that an increase in antioxidant levels activates DNA repair mechanisms, which could lead to a temporarily increased elimination of urinary 8-oxoGuo [42,43]. It has been argued [44,45, W.B. (unpublished results)] that during the first weeks of antioxidant treatment, intracellular 8-oxoGuo is preferentially excreted, followed by a later decrease of urinary excretion after depletion of the initial intracellular oxidized nucleotide pool. In future studies these results should be considered using both intracellular and urinary oxidized DNA metabolites measurement.

There are also no clear references in the published literature as to the possible relationship between the lower 8-oxoGuo values and the elevated vitamin C and E values in both groups by the end of the study. In this regard current data is inconclusive, since on the one hand some researchers have not established a correlation between antioxidant supplementation and elimination of urinary 8-oxoGuo [46], while others have reported an increased elimination of urinary 8-oxoGuo with vitamin C supplementation [47].

Ultimately the lower values at the end of the study remain

an inexplicable outcome, since changes in dietary habits as a possible explanation can also be ruled out. Future studies with larger study groups and over longer time periods could provide greater insight into these relationships.

CONCLUSION

Clearly, the antioxidant nutrients and folate play a major role in maintaining human health, and consumption of fresh fruits and vegetables are promoted as a first line of defense in the prevention of serious illness. This study has shown that, while participants were generally health conscious and consumed vitamin-rich foods, achieving the recommended five portions of fruit and vegetables per day is still difficult for individuals.

Supplementation, in general, proved to be effective for raising plasma levels of all the nutrients studied. Plasma levels of vitamin C reached values associated with a reduced risk for cancer. Selenium levels also correlated with values shown beneficial in other investigations. A supplement derived from dehydrated natural mixed fruit and vegetable juices may, therefore, be beneficial in the prevention of disease and may offer synergistic benefits from nutrient combinations. Further studies into the roles of phytochemicals and other micronutrients found in fruits and vegetables may help us to gain a clearer understanding of the effects and benefits of these foods.

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