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Biomarkers of oxidative stress in overweight men are not influenced by a combination of antioxidants

ELISABET RYTTER[1,2,3], CLARA JOHANSSON[4], BENGT VESSBY[3], ANDERS SJÖDIN[5], LENNART MÖLLER[4], BJÖRN ÅKESSON[6] & SAMAR BASU[1,3]

[1]*Oxidative Stress and Inflammation, and* [2]*Clinical Nutrition and Metabolism, Department of Public Health and Caring Science, Faculty of Medicine, Uppsala University, Sweden,* [3]*Centre of Excellence-Inflammation, Uppsala University Hospital, Uppsala, Sweden,* [4]*Department of Biosciences and Nutrition, Karolinska Institute, Huddinge, Sweden,* [5]*Department of Human Nutrition, Faculty of Life Science, University of Copenhagen, Denmark, and* [6]*Department of Pure and Applied Biochemistry, Biomedical Nutrition, Lund University, Sweden*

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Abstract

The effect of antioxidant supplementation on biomarkers of oxidative stress was investigated in a 6-week intervention study in 60 overweight men. The supplement contained a combination of antioxidants aiming to correspond to the antioxidant content found in a diet rich in fruit and vegetables. Placebo, single or double dose of antioxidants was provided to the subjects. Metabolic variables, plasma antioxidants and biomarkers of oxidative stress (lipid peroxidation and DNA damage) were measured. No effect of supplementation on biomarkers of oxidative stress was observed. Both intervention groups showed substantial increases of plasma antioxidants. This study demonstrated that supplementation with a combination of antioxidants did not affect lipid peroxidation and DNA damage in overweight men, despite increased concentrations of plasma antioxidants. The absence of antioxidant supplement effect might possibly be explained by the chosen study group having a normal level of oxidative stress, duration of the intervention and/or doses of antioxidants.

Keywords: *Antioxidants, oxidative stress, isoprostanes, lipid peroxidation, DNA damage, overweight*

Introduction

Epidemiological studies indicate that a high intake of fruit and vegetables decreases the risk of chronic diseases, e.g. different forms of cancer [1,2] and cardiovascular diseases (CVD) [3]. Dietary recommendations for an increased intake of fruit and vegetables have been established both on international and national levels with the aim to decrease future risk of diseases [4,5]. Different bioactive plant compounds found in fruit and vegetables, such as phytochemicals with antioxidative activity, are suggested to be involved in these beneficial health effects by counteracting oxidative stress in humans [6,7]. Oxidative stress, a condition defined as a disruption of redox signalling and control [8], is suggested to be involved in the development of CVD and cancer [9,10]. A suggested mechanism linking oxidative stress with CVD is an increased oxidation of low density lipoprotein (LDL) [11]. Inhibition of DNA damage caused for example by scavenging of oxidative agents, modulation of detoxification enzymes and regulation of gene expression could explain a potential protective role of antioxidants in reducing the risk of cancer [7]. There are also studies showing that oxidative stress is increased in overweight and obese [12–14]. Fat accumulation in adipocytes causes an increased production of reactive oxygen species and dysregulation of adipocytokines [15]. Overweight individuals have also been documented to have a low intake of antioxidant rich food [16–18]. This could result in an inadequate

Correspondence: Dr S. Basu, Oxidative Stress and Inflammation, Department of Public Health and Caring Sciences, Uppsala University, Uppsala Science Park, SE- 751 85, Sweden. Tel: +46186117958. Fax: +46186117976. Email: samar.basu@pubcare.uu.se

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antioxidant defence that contributes to increased oxidative stress.

Based on the epidemiological data indicating that antioxidants may be involved in decreasing the risk of cancer and CVD, a number of clinical trials have been performed with supplementation of one or a few antioxidants at high doses. The results have been diverse; some studies reported beneficial effects of antioxidants [19,20], others showed no such effect [21,22] and some even observed negative health effects of antioxidant supplementation [23,24]. The different outcome between epidemiological and large intervention studies could be explained by differences in effects of antioxidants originated from fruit and vegetables and antioxidants given as single compounds, respectively. Fruit and vegetables contain a wide range of natural phytochemicals that may act synergistically towards a beneficial effect [7]. In addition, intake of antioxidants in high doses from supplements might disturb the balance between different antioxidants in the body and may have pro-oxidative effects [25].

We hypothesized that supplementation with a combination of several antioxidants at moderate doses could be more beneficial to health since such a combination perhaps better resembles the consumption of antioxidants via a daily intake of fruit and vegetables.

The aim of this study was to investigate to what extent supplementation with a combination of antioxidants would affect the concentration of biomarkers of oxidative stress in overweight middle-aged men.

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Subjects and methods

Study design

The study was a 6-week randomized double-blind, parallel placebo-controlled intervention study performed between April and June. The subjects were randomly divided into three treatment groups; the control group ($n=20$) consuming eight placebo capsules, the single dose group ($n=21$) consuming four capsules with antioxidants and four placebo capsules or the double dose group ($n=19$) consuming eight capsules with antioxidants per day. Measurements were made on two consecutive days at study start and after the 6-week treatment period, respectively. Blood and urine samples were drawn in the morning after an overnight fast. Body height, weight and waist circumference were recorded at the same time. Subjects got oral and written instructions to restrain from alcohol intake and heavy physical activity the day before the clinical examination.

The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving patients were approved by the Ethical Committee of the Medical Faculty at Uppsala University, Sweden (No: Ups 00-045). Written informed consent was obtained from all patients.

Table I. Clinical characteristic of the participants at baseline.*

Variables	Mean (SD)
Age (year)	52 ± 8
Weight (kg)	92.7 ± 10.2
Waist (cm)	100.2 ± 6.9
BMI (kg/m^2)	28.8 ± 2.7
Triacylglycerol (mmol/L)	1.53 ± 0.58
Total cholesterol (mmol/L)	5.92 ± 0.94

*$n=60$.

Subjects

The participants were selected from a group of men responding to an announcement in the local newspaper. A total of 60 subjects were recruited and participated in the study. All subjects underwent clinical assessment and completed a self-administered questionnaire in order to collect data for inclusion and exclusion. Men of 35–65 years old and with a body mass index (BMI) of 25–40 were included. Subjects with diabetes, cancer, inflammatory, thyroid, liver or kidney diseases, as well as smokers, subjects with a high intake of alcohol or with medication that could affect oxidative status were excluded. Antioxidant supplementation was not allowed 1 year prior to the study start. The subjects were instructed to keep their food habits and level of physical activity stable during the study.

Table I shows the baseline clinical characteristics of the participants. Two subjects were classified as outliers for plasma concentrations of α- and β-carotene. The three treatment groups were not significantly different with regard to clinical or biochemical measurement at study start.

Antioxidant supplement

The antioxidant supplements were supplied by Semper AB (Stockholm, Sweden) as capsules. The capsules contained antioxidants mainly extracted from fruits, berries and vegetables (Table II). The extracts were standardized to ensure stable concentrations of specific antioxidative compounds. One or several antioxidants per extract were used for standardization. Four capsules contained amounts of extracts with antioxidant quantities approximately corresponding to 500 g of fruit, vegetables and berries. The placebo capsule contained cellulose and paraffin oil. The production and pre-packing of the capsules were in accordance with Good Manufacturing Practice. The content of antioxidants in the capsules, described in Table II, was verified by analyses both at time of production and at study start. It was not visually possible to identify the capsules by content. Each person received the capsules pre-packed in daily doses labelled with the day of consumption.

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Table II. Characterization of antioxidant supplements used in the study.*

Ingredient/extract	Compound used for standardization	Content/ capsule
Green tea extract	Catechins	40 mg
Vegetable oil concentrate	α-Tocopherol	5 mg
	δ-Tocopherol	8 mg
	β-Tocopherol	0.36 mg
	γ-Tocopherol	22 mg
Rosehip extract	Ascorbic acid	22.5 mg
Rutin	Rutin	15 mg
Grape seed extract	Proanthocyanidins	12 mg
Citrus extract	Flavanones + Flavones	10 mg
Acerola extract	Ascorbic acid	7.5 mg
Cranberry extract	Quinic acid	5.5 mg
Zinc sulphate	Zinc	3.5 mg
Carrot extract	α-Carotene	1 mg
	β-Carotene	2 mg
Bilberry extract	Anthocyanidins	1.5 mg
Marigold extract	Lutein	1 mg
Tomato extract	Lycopene	0.75 mg
Artichoke extract	Cynarin	0.75 mg
Thyme oil	Thymol	30 μg
	Carvacrol	2 μg
Selenium chelate and yeast	Selenium	25 μg
Garlic oil†	Antioxidant not specified	—

*The subjects were randomly divided into three treatment groups. The control group consumed eight placebo capsules per day (containing cellulose and paraffin oil), the single dose group consumed four capsules with antioxidants and four placebo capsules per day, and the double dose group consumed eight capsules with antioxidants per day.
†Derived from 1.5 *g* fresh garlic bulb.

Biochemical measurements

Serum cholesterol and triacylglycerol concentrations were analysed by enzymatic methods in a Monarch 2000 centrifugal analyser (Instrumentation Laboratories, Lexington, MA).

The amounts of α-tocopherol and γ-tocopherol in serum were analysed with high pressure liquid chromatography (HPLC) according to Öhrvall et al. [26] and adjusted for the sum of the cholesterol and the triacylglycerol concentrations [27]. The carotenoids were analysed by adding ethanol to serum in order to precipitate proteins. Thereafter the carotenoids, α-carotene, β-carotene, lycopene and lutein, were extracted into hexane and evaporated under nitrogen to dryness. The residue was re-dissolved in ethanol and the carotenoids were detected by HPLC with a diode array detector using a Chromolith Performance column (MERCK, Darmstadt, Germany). Mobile phase for the analysis was acetonitrile/dichloromethane/methanol (72.5:2.5:25). The plasma selenium concentration was measured by electrothermal atomic absorption spectrometry with Zeeman background correction [28]. The selenoprotein P concentration in plasma was measured with a radioimmunoassay as described elsewhere [29]. The concentration of selenoprotein P was expressed in arbitrary units relative to a standard of pooled plasma.

Free 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) was analysed in urine by a validated radioimmunoassay developed by Basu [30]. The intra-assay CV was 14.5% at low concentrations and 12.2% at high concentrations. 8-Iso-$PGF_{2\alpha}$ concentrations were adjusted by creatinine values to correct for variations in the glomerular filtration rate. Urinary creatinine concentrations were determined by using IL Test creatinine, 181672-00 in a Monarch 2000 centrifugal analyser (Instrument Laboratories, Lexington, MA). The plasma malondialdehyde (MDA) concentration was measured by HPLC and fluorescence detection as earlier described [31]. The level of 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-oxodG) per undamaged dG was analysed by HPLC with electrochemical and ultraviolet detection as previously described by Hofer and Möller [32]. Oxidatively damaged purines, specifically formamido pyrimidine-DNA glycosylase (FPG)-sensitive sites, were analysed by the Comet assay as previously described by Johansson et al. [33].

Statistical analysis

Statistical analyses were carried out using the statistical software JMP version 3.2 (SAS Institute, Cary, NC). Variables with skewed distribution (Shapiro-Wilks W-test <0.95) were log-transformed before statistical analysis. If variables were not normally distributed after logarithmic transformation, non-parametric tests were used. All tests were two-tailed. Treatment groups were compared by one-way ANOVA test or Wilcoxon´s rank sum test followed by unpaired *t*-test or Wilcoxon 2-sample test when required. The Pearson's or Spearman's coefficient was calculated when analysing correlations. p-values <0.05 were considered significant.

Results

No differences between any of the three groups, with regard to changes in levels of lipid peroxidation (8-iso-$PGF_{2\alpha}$ and MDA) or DNA damage (8-oxodG and FPG-sensitive sites), were observed after 6 weeks of antioxidant intervention (Table III). No differences in change of BMI, weight, triacylglycerol or cholesterol were observed between the three groups.

Both the single and double dose groups showed substantial increases of all measured plasma antioxidants compared to the control group, except for α-tocopherol (Table IV). The increased concentrations of γ-tocopherol, α-carotene, β-carotene, lycopene and selenoprotein P were also higher in the double dose group than in the single dose group. The increased concentrations of lutein and selenium were

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Table III. Biomarkers of oxidative stress at baseline and after 6 weeks of intervention in the three treatment groups.*

Biomarker of oxidative stress		Baseline *M* (SD)	6 weeks *M* (SD)	Absolute change *M* (SD)	*p* for difference between groups†
8-iso-PGF$_{2\alpha}$	Placebo	0.14 ± 0.03	0.18 ± 0.07	0.037 ± 0.057	
(nmol/mmol Cr)	Single dose	0.15 ± 0.04	0.17 ± 0.08	0.020 ± 0.068	0.26
	Double dose	0.15 ± 0.05	0.17 ± 0.13	0.025 ± 0.092	0.20
MDA‡	Placebo	0.56 ± 0.09	0.54 ± 0.13	–0.020 ± 0.111	
(μmol/L)	Single dose	0.55 ± 0.13	0.51 ± 0.16	–0.042 ± 0.123	0.45
	Double dose	0.52 ± 0.09	0.53 ± 0.16	0.001 ± 0.150	0.77
8-oxodG	Placebo	0.67 ± 0.29	0.66 ± 0.23	–0.009 ± 0.323	
(8-oxodG/10^6 dG)	Single dose	0.77 ± 0.43	0.82 ± 0.31	0.050 ± 0.333	0.58
	Double dose	0.76 ± 0.28	0.71 ± 0.38	–0.048 ± 0.467	0.59
FPG-sensitive sites	Placebo	27.4 ± 6.0	29.9 ± 4.0	2.42 ± 7.70	
(tail moment)	Single dose	28.0 ± 5.8	28.9 ± 6.4	0.90 ± 7.98	0.54
	Double dose	27.6 ± 6.2	26.4 ± 3.3	–1.18 ± 6.79	0.13

*Control group $n=20$, single dose group $n=21$, double dose group $n=19$.
†Comparison between single or double dose group and control group.
‡59 subjects
8-iso-PGF$_{2\alpha}$= 8-iso-prostaglandin F$_{2\alpha}$; Cr = creatinine; MDA = malondialdehyd; 8-oxodG = 8-oxo-7,8-dihydro-2′-deoxyguanosine; FPG = formamido pyrimidine glycosylase.

not significantly different between the single and double group.

Excluding the two subjects, classified as outliers for α- and β-carotene, did not alter the results of the intervention. Thus, all presented data and statistical analyses include 60 subjects.

Discussion

In the present study, we found no effects of supplementation with a combination of antioxidants aiming to correspond to the antioxidant content found in a diet rich in fruit and vegetables, on markers of lipid

Table IV. Plasma concentrations of antioxidants at baseline and after 6 weeks of intervention in the three treatment groups.*

Antioxidant		Baseline *M* (SD)	6 weeks *M* (SD)	Absolute change *M* (SD)	*p* for difference between groups†
α-Tocopherol	Placebo	1.25 ± 0.16	1.25 ± 0.21	0.004 ± 0.080	
(mg/mmol)	Single dose	1.19 ± 0.16	1.20 ± 0.21	0.019 ± 0.100	0.60
	Double dose	1.26 ± 0.20	1.29 ± 0.17	0.026 ± 0.116	0.48
γ-Tocopherol	Placebo	0.09 ± 0.03	0.10 ± 0.02	0.013 ± 0.031	
(mg/mmol)	Single dose	0.09 ± 0.04	0.16 ± 0.06	0.067 ± 0.047‡,¶	<0.0001
	Double dose	0.09 ± 0.04	0.22 ± 0.05	0.131 ± 0.053‡,¶	0.0008
α-Carotene§ (mg/L)	Placebo	0.11 ± 0.10	0.08 ± 0.06	–0.027 ± 0.063	
	Single dose	0.06 ± 0.03	0.12 ± 0.04	0.057 ± 0.026‡,¶	<0.0001
	Double dose	0.07 ± 0.03	0.32 ± 0.11	0.258 ± 0.096‡,¶	<0.0001
β-Carotene (mg/L)	Placebo	0.34 ± 0.27	0.23 ± 0.15	–0.111 ± 0.136	
	Single dose	0.24 ± 0.17	0.32 ± 0.31	0.087 ± 0.168‡,¶	<0.0001
	Double dose	0.23 ± 0.11	0.58 ± 0.26	0.347 ± 0.178‡,¶	<0.0001
Lycopene (mg/L)	Placebo	0.27 ± 0.12	0.21 ± 0.09	–0.061 ± 0.069	
	Single dose	0.23 ± 0.13	0.25 ± 0.10	0.017 ± 0.075‡,¶	<0.0001
	Double dose	0.27 ± 0.10	0.36 ± 0.10	0.093 ± 0.097‡,¶	<0.0001
Lutein (mg/L)	Placebo	0.16 ± 0.07	0.14 ± 0.07	–0.021 ± 0.036	
	Single dose	0.15 ± 0.08	0.18 ± 0.07	0.028 ± 0.039‡	<0.0001
	Double dose	0.17 ± 0.06	0.24 ± 0.08	0.070 ± 0.049‡	<0.0001
Selenium (μg/L)	Placebo	91.7 ± 21.5	93.2 ± 19.9	1.52 ± 7.91	
	Single dose	96.1 ± 21.6	110.7 ± 24.6	14.67 ± 12.04‡	0.0002
	Double dose	94.1 ± 17.5	114.5 ± 22.6	20.40 ± 16.41‡	<0.0001
SeP (a.u.)	Placebo	1.29 ± 0.13	1.27 ± 0.13	–0.012 ± 0.103	
	Single dose	1.30 ± 0.17	1.46 ± 0.18	0.159 ± 0.118‡,¶	<0.0001
	Double dose	1.32 ± 0.22	1.59 ± 0.24	0.271 ± 0.202‡,¶	<0.0001

*Control group $n=20$, single dose group $n=21$, double dose group $n=19$.
†Comparison between single or double dose group and control group.
‡Significant difference between the control group and the single or double dose group ($p < 0.05$).
¶Significant difference between the single dose group and the double dose group ($p < 0.05$).
§50 subjects.
SeP = selenoprotein P; a.u. = arbitrary units.

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peroxidation and DNA damage in spite of increased antioxidant concentrations in plasma. These results do not support the hypothesis that supplementation with a combination of antioxidants at moderate doses could be more beneficial for health than supplementation with one or a few antioxidants in high doses.

In order to study the effects of antioxidant supplementation it is reasonable to investigate subjects where a somewhat decreased antioxidative capacity and/or enhanced oxidative stress could be expected. Overweight subjects are a potential risk group who might have an increased level of oxidative stress [12,14]. Consumption of fruit and vegetables in Swedish men amounts to only half of the recommended 500 g of fruit and vegetables [34]. Consequently overweight men could be a group who are likely to benefit from antioxidant supplementation. In other studies, small or no effects of antioxidant supplementation (specifically α-tocopherol and vitamin C) on isoprostanes have been shown in healthy subjects [35–37]. However, a pronounced decrease on the concentration of isoprostanes has been observed in diabetic and hypercholesterolaemic patients as well as overweight smokers [38–40]. Studies investigating the effect of an intake of fruit and vegetable products on isoprostanes concentrations have shown diverse outcome. The 6-a-day study from Denmark, where healthy subjects consumed daily six servings of fruit and vegetables [41] and another study supplementing male smokers with a vegetable burger and a fruit drink rich in antioxidants [42], did not alter the levels of 8-iso-$PGF_{2\alpha}$, while 500 ml high-pressurized orange juice reduced the level of isoprostanes in healthy subjects [43]. One study, investigating the effect of fruit and vegetable intake in women at risk of breast cancer, observed decreased concentrations of both 8-iso-$PGF_{2\alpha}$ and 8-oxodG concentrations, while the MDA level was unaffected [44]. A review by Basu and Helmersson [45] concludes that fruits, vegetables and tea do not generally change the basal 8-iso-$PGF_{2\alpha}$ formation. In a previous study, beneficial effects on MDA were only observed in the group with the highest initial concentration of MDA when using multi-antioxidant supplementation [46]. An additional study in which a health-conscious population were advised to eat at least 400 g of fruit and vegetables for 12 weeks failed to find any effect on MDA [47]. Møller and Loft [48] summarized that ingestion of antioxidants could be associated with reduced levels of damaged DNA, but no relationship was found between type of antioxidant used and beneficial effect. To summarize, intervention studies investigating the effects of antioxidant supplementation or intake of fruit and vegetables on biomarkers of lipid peroxidation and DNA damage present various results and effects. This could partly be due to the differences between study subjects where patients with elevated levels of certain biomarkers of oxidative stress appear to be more likely to benefit from an increased intake of antioxidants. The concentrations of the investigated biomarkers of oxidative stress (8-iso-$PGF_{2\alpha}$, MDA and 8-oxodG) in our subjects were found to be in the same range as previously found in healthy control groups [30,49,50] except for FPG-sites. The level of FPG-sites was higher in the current study compared to the level found in healthy subjects investigated by Hofer et al. [50]. These comparisons indicate that our subjects have normal levels of oxidative stress biomarkers which might partly explain the absence of a beneficial effect of antioxidant supplementation. The absence of effect could also be due to the choice of biomarkers of oxidative stress. Even if four different biomarkers were studied, they might all have been unsuitable for the investigation.

The duration and the dose of antioxidants used in an intervention study are believed to influence the possible effects on oxidative stress *in vivo* [51]. In a recent intervention study of 20 weeks duration, Roberts et al. [52] reported that an association exists between the dose of vitamin E (0-3200 IU) and change in concentration of isoprostanes among individuals with hypercholesterolemia. A reduction of F_2-isoprostane was observed at high doses of vitamin E ($\geq$1600 IU), but it did not occur until 16 weeks of supplementation. Other studies [35,53] investigating vitamin E supplementation in lower doses and under shorter duration than used by Roberts et al. observed no effect on F_2-isoprostane concentration. These results also raise the question if the absence of beneficial effects in the present study partly could be due to the duration of the intervention and the chosen doses of antioxidant.

A limitation of the study design was the absence of a control group of normal weight. There is also a lack in the literature of identification criteria to select subjects that may benefit from antioxidant supplementation. This usually makes it difficult to design an appropriate study with antioxidant intervention. However, an innovative aspect of this study was the development of a supplement containing a broad combination of extracts derived from fruit, berries and vegetables aiming to correspond to the antioxidant content found in a diet rich in fruit and vegetables. Also, according to our knowledge, no other study has previously investigated the effect of antioxidant supplementation on four different biomarkers of oxidative stress simultaneously.

In conclusion, the results indicate that a daily supplementation with a combination of antioxidants approximately corresponding to the content which may be found in a recommended diet rich in fruit and vegetables does not show any protective effect on lipid peroxidation or DNA damage in overweight men, despite considerably increased concentrations of plasma antioxidants. The absence of effect might possibly be explained by the duration of the intervention, the

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doses of antioxidants and/or the selection of the study group having a normal level of oxidative stress.

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