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Supplementation with a combination of antioxidants does not affect glycaemic control, oxidative stress or inflammation in type 2 diabetes subjects

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Abstract

The present clinical trial examined the influence of a supplement, containing a combination of antioxidants extracted from fruit, berries and vegetables, on levels of plasma antioxidants (tocopherols, carotenoids and ascorbate), glycaemic control (blood glucose, HbA1c, insulin), oxidative stress biomarkers (F_2-isoprostane, malondialdehyd, nitrotyrosine, 8-oxo-7, 8-dihydro-2′-deoxyguanosine, formamidopyrimidine glycosylase sites, frequency of micronucleated erythrocytes) and inflammatory markers (interleukin-6, C-reactive protein, prostaglandin $F_{2\alpha}$-metabolite) in type 2 diabetes. Forty subjects were randomly assigned to control, single or double dose group and completed the study. In summary, 12 weeks of antioxidant supplementation did neither affect glycaemic control nor the levels of biomarkers of oxidative stress or inflammation, despite substantially increased plasma concentrations of antioxidants. The absence of an effect may be explained by the selected study subjects with relatively well-controlled diabetes, a high intake of fruit and vegetable and levels of plasma antioxidants, biomarkers of oxidative stress and inflammatory markers comparable to those found in healthy subjects.

Keywords: *Antioxidants, supplementation, glycaemic control, oxidative stress, inflammation, diabetes mellitus type 2*

Introduction

Type 2 diabetes is a disease characterized by a metabolic imbalance and associated with increased oxidative stress and low-grade inflammation [1,2]. Patients with type 2 diabetes have been reported to have an increased level of lipid peroxidation [3,4], oxidative DNA damage [5,6] and protein oxidation [7]. This probably results from hyperglycaemia that leads to an increased *in vivo* production of reactive oxygen species (ROS) [8]. At the same time there are indications that a defect in the anti-oxidative protection may occur which might cause an impaired defence against oxidative damage of biological compounds [9]. Elevated concentrations of inflammatory markers are also observed in subjects with type 2 diabetes [10–12].

Epidemiological studies have observed an association between a high intake of vegetables, high intake of dietary antioxidants as well as use of vitamin supplement and a reduced risk for diabetes [13–16]. The hypothesis that antioxidants might contribute to a reduced risk to develop diabetes, improvement of glycaemic control or reduction of oxidative stress or inflammation has not been sufficiently supported by interventional studies [3,17–26]. These studies generally used single antioxidants in high doses that might disturb the balance between different antioxidants in

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the body and that could even have pro-oxidative effects [27,28]. This is in contrast to the wide range of natural antioxidants found in fruits and vegetables that may act synergistically in a healthy approach [28,29].

We therefore hypothesized that a supplement, containing a combination of several natural antioxidants extracted from fruit and vegetables, supplied in moderate doses, might act more beneficially to human health than single antioxidants in high doses. Our previous study using a similar intervention model suggested a possible beneficial effect of supplementation of such a combination of antioxidants in a group of overweight men [30]. To increase the possibilities to achieve clear effects we doubled the dose of supplementation in this study and included patients with type 2 diabetes, a group of subjects that could be expected to be at risk for elevated oxidative stress and inflammation. We have earlier reported that there are negative correlations between the habitual intake of fruit and vegetables and dietary antioxidants on the one hand and biomarkers of both oxidative stress and inflammation on the other, in diabetic subjects [31]. We also included additional measurements of glycaemic control, inflammatory markers and a larger number of biomarkers of oxidative stress.

The aim of the present study was to investigate if supplementation with a combination of antioxidants, extracted from fruit and vegetables, would affect the concentrations of plasma antioxidants, glycaemic control and biomarkers of oxidative stress and inflammation in subjects with type 2 diabetes.

Material and methods

Subjects

The participants were recruited through advertisement in a local newspaper (Uppsala, Sweden). A total of 47 subjects were enrolled in the study. All subjects completed a self-administered questionnaire and underwent clinical assessment before inclusion in the study. Instructions were given to all subjects to keep their food habits and the level of physical activity stable during the test period. The subjects were instructed to stop any antioxidant supplementation at least 1 month before the start of the study. The participants were to have type 2 diabetes treated with either diet or diet and oral hypoglycaemic medication, glycosylated haemoglobin (HbA1c) $< 10\%$, an age of 40–75 years, body mass index (BMI) < 35 kg/m^2 and a stable body weight during the last 3 months before entering the study. However, two subjects with BMI > 35 kg/m^2 but ≤ 37 kg/m^2 were included in the study as they in all other aspects fulfilled the inclusion criteria. Subjects with insulin-dependent diabetes, cancer, known cardiovascular disease, acute inflammatory, thyroid, liver or kidney disease were excluded, as well as subjects with a high intake of alcohol or with medication that could affect antioxidative, oxidative or inflammatory status.

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. Written informed consents were obtained from all patients in the study.

Study design

The intervention study had a parallel randomized double-blind and placebo-controlled design. The study was performed during 12 weeks between March and June, followed by an 8-week washout period. The participants were randomly divided into three test groups. The control group consumed 16 placebo capsules, the single dose group consumed eight antioxidant capsules and eight placebo capsules and the double dose group consumed 16 antioxidant capsules per day. Eight capsules were consumed at breakfast and dinner, respectively.

Sample and data collection

Data was obtained at study start, after the 12-week treatment period and after the 8-week washout period. Blood and urine samples were drawn in the morning after an overnight fast. Anthropometric measurements were performed and systolic and diastolic blood pressures were recorded at the same time. Subjects received both oral and written instructions of the importance of refraining from heavy physical activity and alcohol intake 24 h prior to data collection.

Antioxidant supplement

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

Dietary intake

A dietary survey was performed 1 week before study start and after 3 weeks of intervention. It included a

Table I. Characterization of antioxidant supplement used in the study.*

Ingredient/extract	Compound used for standardization Antioxidant	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 16 placebo capsules per day (containing paraffin oil), the single dose group consumed eight capsules with antioxidants and eight placebo capsules per day and the double dose group consumed 16 capsules with antioxidants per day.
†Derived from 1.5 *g* fresh garlic bulb.

pre-coded 3-day record book. Subjects were instructed to record everything they ate during 2 weekdays and 1 day during the weekend. The record book was a shorter modified version of a pre-coded 7-day record book called 'Menyboken', validated [32] and used in two nationwide dietary surveys in Sweden [33,34]. The food records were analysed with a computer program estimating the intake of various food-groups as well as the intake of macro- and micronutrients. Vegetables, root-crops, fruits, berries, marmalade, jam and stews and preserves made of fruits, berries and root-crops were included in the fruit and vegetable intake.

Assays and biochemical measurements

Anthropometry and metabolic control. All anthropometric parameters were measured while the subjects wore light indoor clothes but no shoes. The body weight was measured using a digital scale to the nearest 0.1 kg and height to the nearest whole cm. The waist circumference (waist) in cm was assessed midway between the lowest rib and the iliac crest, in a supine position, using a non-stretchable tape measure. Systolic and diastolic blood pressures were measured with an Aneroid Sphygmomanometer Blood Pressure Cuff (Umedico, 12 × 35) in a supine position after 5 min rest. Blood glucose concentration was assessed using an enzymatic technique (HemoCue). HbA1c was assayed with high performance liquid chromatography (HPLC). Plasma insulin was analysed in a Coda Automated EIA Analyser (Bio-Rad Laboratories, California, USA) by an enzymatic immunological assay (Mercodia, Uppsala, Sweden). Serum cholesterol, high density lipoprotein (HDL) cholesterol and triacylglycerol concentrations were measured in a Konelab 20 Clinical Chemistry Analyser (Thermo Electron Corporation, Vantaa, Finland) by enzymatic colourimetric methods (Thermo Electron Corporation). The concentration of low density lipoprotein (LDL) cholesterol was calculated according to Friedewald et al. [35].

Antioxidants. The concentrations of 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 identified 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 serum concentrations of α-tocopherol and γ-tocopherol were analysed with HPLC according to Öhrvall et al. [36] and adjusted for the sum of triacylglycerol and cholesterol concentrations [37]. Intra-assay coefficient of variation (CV) for the method was 4.5% for α-tocopherol and 7.2% for γ-tocopherol.

Ascorbic acid concentration in plasma was measured fluorimetrically after oxidation using ascorbate oxidase and derivatization with 1-orthophenylenediamine [38,39]. Samples were stabilized by metaphosphoric acid and stored at 70°C before analysis. Intra-assay CV for the method was ~ 4% and inter-assay CV ~ 5%. The detection limit was ~ 5 μmol/L.

Oxidative stress biomarkers. Urinary concentrations of free 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), a major F_2-isoprostane, were analysed using a validated radioimmunoassay (RIA) developed by Basu [40]. The intra-assay CV was 14.5% at low concentrations and 12.2% at high concentrations. The cross-reactivity of the 8-iso-$PGF_{2\alpha}$ antibody with 15-keto-13,14-dihydro-8-iso-$PGF_{2\alpha}$, 8-iso-$PGF_{2\beta}$, $PGF_{2\alpha}$, 15-keto-$PGF_{2\alpha}$, 15-keto-13,14-dihydro-$PGF_{2\alpha}$, thromboxane B_2, 11β-$PGF_{2\alpha}$, 9β-$PGF_{2\alpha}$ and 8-iso-$PGF_{3\alpha}$ was 1.7, 9.8, 1.1, 0.01, 0.01, 0.1, 0.03, 1.8 and 0.6%, respectively. The detection limit of the assay was 23 pmol/L.

The urinary levels of 8-iso-$PGF_{2\alpha}$ were adjusted for urinary creatinine concentration.

Plasma concentrations of malondialdehyd (MDA) were analysed by HPLC and fluorescence detection including thiobarbituic acid reaction [41].

The concentration of plasma nitrotyrosine was assayed by a commercially available enzymatic immunological assay (Bioxytech, OxisResearch, Portland, USA).

For the 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) analyses, DNA was isolated using a cold work-up procedure with small modifications, followed by enzymatic hydrolysis [42,43]. The amount of 8-oxodG per undamaged 2′-deoxyguanosine (dG) was assessed using on-line electrochemical and ultraviolet detection after separation of the nucleosides with HPLC.

Formamidopyrimidine glycosylase (FPG) sites were analysed in DNA from lymphocytes and monocytes with a high alkaline FPG version of the comet assay [44] with some modifications.

A flow cytometry-based micronucleus assay was used for the analysis of frequency of micronucleated transferrin-positive reticulocytes (fMN-Trf-Ret) [45] measuring chromosomal damages. The very young erythrocytes were separated from the mature erythrocytes before analysis since the micronuclei are formed in the bone marrow at the last cell division of the erythroblasts. The detection limit was approximately a difference of 0.2 of the background fMN-Trf-Ret and the background fMN-Trf-Ret was ~ 1‰.

Inflammatory biomarkers. High sensitivity CRP (hsCRP) measurements were performed by latex-enhanced reagent (Dade Behring, Deerfield, IL) with the use of a Behring BN ProSpec analyser (Dade Behring). The intra-assay CV of the hsCRP method was 1.4% at both 1.23 mg/L and 5.49 mg/L.

Interleukin 6 (IL-6) in plasma was assessed using a high sensitivity ELISA kit (IL-6 HS, R&D Systems, Minneapolis, MN). Standards and samples were pipetted into a microtiter plate coated with monoclonal antibody against IL-6. After incubation and washing, enzyme substrate solution was pipetted and followed by anti-IL-6 antibody. The colour reaction was proportional to the bound IL-6. The total CV of the method was 7% and inter-assay CV was 5%.

15-Keto-dihydro-prostaglandin $F_{2\alpha}$ (15-keto-dihydro-$PGF_{2\alpha}$), a major metabolite of primary $PGF_{2\alpha}$, was measured in urine, by a validated RIA developed by Basu [46]. The intra-assay CV was 12.2% at low concentrations and 14.0% at high concentrations. The cross-reactivity of the antibody with $PGF_{2\alpha}$, 15-keto-$PGF_{2\alpha}$, PGE_2, 15-keto-13,14-dihydro-PGE_2, 8-iso-15-keto-13,14-dihydro-$PGF_{2\alpha}$, 11β-$PGF_{2\alpha}$, 9β-$PGF_{2\alpha}$, TXB_2 and 8-iso-$PGF_{3\alpha}$ was 0.02, 0.43, < 0.001, 0.5, 1.7, < 0.001, < 0.001, < 0.001 and 0.01%, respectively. The detection limit was 45 pmol/L. Concentrations were corrected for urinary creatinine levels.

Other assays. Creatinine concentration was analysed in urine samples by a colourimetric method using IL Test creatinine, 181672-00 in a Monarch 2000 centrifugal analyser (Instrument Laboratories, Lexington, MA).

Statistical analyses

P-values less than 0.05 were considered as significant. All tests were two-tailed. Variables with skewed distribution (Shapiro Wilks W-test > 0.95) were logarithmically transformed before analyses. Non-parametric tests were used for variables, not normally distributed after logarithmic transformation. Differences between treatment groups were determined by one-way ANOVA test or Wilcoxon's rank sum test followed by unpaired *t*-test or Wilcoxon 2-sample test when required.

Results

Out of 47 subjects, 40 completed the study. Reasons for withdrawal were gastrointestinal problems, nausea, lack of time or ear disorders. Excluding one subject classified as an outlier for triacylglycerides did not alter the results of the intervention. The statistical analyses were performed with and without two subjects with hsCRP > 10 mg/l since studies investigating low-grade inflammation use a maximum CRP-level of 10 mg/l to eliminate the influence of acute inflammatory diseases. The two analyses did not show any differences in results. All presented data and statistical analyses therefore include 40 subjects.

Baseline characteristics are presented in Table II. The three groups were well matched except for age. The mean age in the double dose group was somewhat higher than in the single dose and control groups. Differences between sex were found for plasma α-carotene, β-carotene, lutein and ascorbate (women > men), weight and waist (men > women) and cholesterol, HDL- and LDL-cholesterol (women > men). Women had higher values of two oxidative stress biomarkers (8-iso-$PGF_{2\alpha}$ and nitrotyrosine) than men, while men had a higher level of the inflammatory indicator IL-6.

The dietary survey was performed to examine if the participants food habits were influenced and changed by taking part in an interventional study. No differences concerning mean energy intake, the energy intake from fat, protein and carbohydrate, fibre intake or intake of fruit and vegetables were observed between the three groups at baseline, except a somewhat higher intake of fruit and vegetables in the double dose group compared to the control group.

Table II. Baseline characteristics of the participants.

Variable	Mean or No	SD
Subjects (women/men)	40 (22/18)	
Non-smokers/smokers	34/6	
Diabetic treatment; diet/diet + medication	16/24	
Age (years)	61.9	7.2
Weight (kg)*	81.6	14.9
Waist (cm)*	99.0	11.2
BMI (kg/m^2)	28.3	3.8
Systolic blood pressure (mmHg)	142.0	12.0
Diastolic blood pressure (mmHg)	76.1	9.1
Triacylglycerol (mmol/L)	1.59	0.82
LDL-cholesterol (mmol/L)*	2.93	0.88
HDL-cholesterol (mmol/L)*	1.08	0.23
Cholesterol (mmol/L)*	4.72	0.97

*Significant differences ($p < 0.05$) between sex using unpaired *t*-test or Wilcoxon 2-sample test.

The average intake of fruit and vegetables at the start of the study (464 g/day) was in line with the Swedish food-based dietary guidelines recommending 500 g of fruit and vegetables per day [47]. No change of food habits was observed during the intervention when comparing the three groups.

Except for α-tocopherol, both the single and double dose groups showed increases of all measured plasma antioxidant concentrations during the intervention compared to the control group (Table III). The increase of γ-tocopherol and lycopene concentrations was higher in the double dose group than in the single dose group following treatment. The increased concentrations of lutein, α-carotene, β-carotene and ascorbate were, however, not significantly different between the groups receiving active treatment.

No changes of glycaemic control (Table IV), BMI or blood lipids were observed in the single and double dose groups compared with the control group, except for a decreased level of HDL-cholesterol in the single dose group during the intervention compared to the control group. Neither were there any changes of biomarkers of oxidative stress (8-iso-PGF$_{2\alpha}$, MDA, nitrotyrosine, 8-oxodG, FPG-sites, fMN-Trf-Ret) and inflammation (hsCRP, IL-6, 15-keto-dihydro-PGF$_{2\alpha}$) in subjects receiving antioxidant supplement (single and double dose groups) vs placebo after 12 weeks of antioxidant treatment (Table V). Adjustment for potential confounders (weight change, HbA1c, age, sex and baseline values) did not alter these observations.

The levels of α-carotene and β-carotene were still increased in the groups receiving active treatment vs the control group after 8 weeks of wash-out. No changes of oxidative stress and inflammatory biomarkers were observed in subjects receiving antioxidant supplement compared to placebo also at the end of the washout period. Insulin, ascorbate, MDA, nitrotyrosine and fMN-Trf-Ret were not measured after the washout phase.

Discussion

In the present study we designed an antioxidant supplement based on the hypothesis that a number of naturally occurring antioxidants, extracted from

Table III. Plasma concentrations of antioxidants at baseline and after 12 weeks of intervention in the three treatment groups.*

Variable		Baseline *M* (SD)	12 weeks *M* (SD)	Absolute change *M* (SD)	*p* for difference between groups†
α-Tocopherol (mg/mmol)	Control	2.00 (0.24)	2.06 (0.24)	0.053 (0.241)	
	Single dose	1.93 (0.24)	1.89 (0.35)	−0.038 (0.336)	ns
	Double dose	2.01 (0.31)	2.12 (0.32)	0.105 (0.110)	ns
γ-Tocopherol (mg/mmol)	Control	0.10 (0.04)	0.11 (0.03)	0.005 (0.024)	
	Single dose	0.10 (0.04)	0.30 (0.09)	0.199 (0.061)‡	< 0.0001
	Double dose	0.09 (0.04)	0.40 (0.10)	0.310 (0.088)‡	< 0.0001
α-Carotene (mg/L)	Control	0.13 (0.26)	0.09 (0.18)	−0.035 (0.083)	
	Single dose	0.07 (0.05)	0.36 (0.15)	0.282 (0.138)	< 0.0001
	Double dose	0.14 (0.16)	0.46 (0.21)	0.317 (0.159)	< 0.0001
β-Carotene (mg/L)	Control	0.35 (0.69)	0.22 (0.40)	−0.127 (0.287)	
	Single dose	0.19 (0.12)	0.62 (0.35)	0.431 (0.283)	< 0.0001
	Double dose	0.33 (0.31)	0.88 (0.49)	0.551 (0.334)	< 0.0001
Lycopene (mg/L)	Control	0.16 (0.07)	0.14 (0.05)	−0.021 (0.047)	
	Single dose	0.22 (0.08)	0.33 (0.10)	0.115 (0.057)‡	< 0.0001
	Double dose	0.14 (0.08)	0.31 (0.13)	0.172 (0.078)‡	< 0.0001
Lutein (mg/L)	Control	0.10 (0.02)	0.11 (0.04)	0.005 (0.040)	
	Single dose	0.12 (0.06)	0.31 (0.17)	0.198 (0.155)	< 0.0001
	Double dose	0.12 (0.06)	0.37 (0.27)	0.242 (0.232)	< 0.0001
Ascorbate (μM)	Control	33.72 (11.43)	35.86 (15.01)	2.139 (7.529)	
	Single dose	28.84 (12.51)	53.20 (12.86)	24.362 (14.354)	< 0.0001
	Double dose	36.31 (9.59)	58.48 (10.34)	22.164 (14.043)	0.0001

*Control group, $n = 13$, single dose group, $n = 13$, double dose group, $n = 14$.
†Comparison between single or double dose group and control group.
‡Significant difference between the single dose group and the double dose group ($p < 0.05$).

Table IV. Biomarker for glycaemic control at baseline and after 12 weeks of intervention in the three treatment groups.*

Variable		Baseline *M* (SD)	12 weeks *M* (SD)	Absolute change *M* (SD)	*p* for difference between groups
HbA_{1c} (%)†	Control	6.49 (0.83)	6.68 (0.95)	0.192 (0.350)	
	Single dose	6.01 (1.01)	6.18 (1.22)	0.177 (0.383)	ns
	Double dose	5.96 (0.63)	5.92 (0.65)	−0.031 (0.405)	ns
Blood glucose (mmol/L)	Control	8.76 (2.20)	8.37 (1.73)	−0.392 (1.011)	
	Single dose	8.37 (2.95)	7.88 (2.32)	−0.492 (1.929)	ns
	Double dose	7.16 (1.41)	7.16 (1.04)	0.000 (0.760)	ns
Insulin (mU/L)	Control	11.25 (5.68)	10.91 (5.30)	−0.346 (2.720)	
	Single dose	13.08 (7.45)	12.00 (6.10)	−1.084 (5.227)	ns
	Double dose	9.49 (6.25)	7.83 (4.66)	−1.657 (3.599)	ns

*Control group, $n = 13$, single dose group, $n = 13$, double dose group, $n = 14$.
†39 subjects.

fruit and vegetables, in moderate doses might act more beneficially than supplements containing high doses of a single antioxidant. The goal was to investigate the effects of daily supplementation of a combination of antioxidants on plasma antioxidants, glycaemic control and markers of oxidative stress and inflammation in a group of subjects with type 2 diabetes. We found that 12 weeks of antioxidant supplementation in general markedly increased plasma concentrations of antioxidants. Contrary to our hypothesis, however, the increases in plasma antioxidants were not associated with improved glycaemic control or alterations of oxidative stress or inflammatory biomarkers.

Previous studies of supplementation with different dietary antioxidants in subjects with type 2 diabetes have shown diverging effects on the same parameters. Six weeks of supplementation with 500 mg of mixed tocopherols reduced the level of plasma F_2-isoprostanes but had no effect on urinary F_2-isoprostanes, inflammatory

Table V. Biomarkers of oxidative stress and inflammation at baseline and after 12 weeks of intervention in the three treatment groups.*

Variable		Baseline *M* (SD)	12 weeks *M* (SD)	Absolute change *M* (SD)	*p* for difference between groups
Oxidative stress					
8-iso-$PGF_{2\alpha}$ (nmol/mmol ceatinine)	Control	0.22 (0.14)	0.20 (0.11)	−0.018 (0.049)	
	Single dose	0.20 (0.07)	0.20 (0.08)	−0.006 (0.066)	ns
	Double dose	0.17 (0.07)	0.18 (0.06)	0.008 (0.036)	ns
MDA† (μmol/L)	Control	0.66 (0.09)	0.62 (0.07)	−0.043 (0.084)	
	Single dose	0.67 (0.08)	0.65 (0.06)	−0.034 (0.061)	ns
	Double dose	0.71 (0.09)	0.68 (0.09)	−0.025 (0.077)	ns
Nitrotyrosine (nmol/L)†	Control	348.8 (492.7)	362.9 (490.6)	14.08 (183.92)	
	Single dose	197.2 (311.8)	144.8 (186.1)	−52.46 (154.12)	ns
	Double dose	198.0 (401.7)	193.9 (404.1)	−4.08 (16.10)	ns
8-oxodG† (8-oxodG/10^6 dG)	Control	1.00 (0.67)	1.56 (2.88)	−0.242 (0.738)	
	Single dose	0.97 (0.36)	0.89 (0.34)	−0.082 (0.496)	ns
	Double dose	0.84 (0.34)	1.05 (1.16)	0.207 (1.183)	ns
FPG-sites (% tail)	Control	28.98 (15.98)	26.26 (7.66)	−2.716 (14.742)	
	Single dose	31.67 (13.69)	22.59 (5.82)	−9.083 (15.680)	ns
	Double dose	31.00 (13.26)	26.38 (6.10)	−4.621 (15.255)	ns
Frequency of MN-Trf-Ret† (‰)	Control	1.08 (0.58)	1.25 (0.54)	0.114 (0.349)	
	Single dose	0.91 (0.56)	1.08 (0.89)	0.169 (0.426)	ns
	Double dose	0.73 (0.30)	0.77 (0.31)	0.046 (0.186)	ns
Inflammation					
hsCRP (mg/L)‡	Control	1.68 (1.032)	1.50 (1.10)	−0.182 (0.659)	
	Single dose	2.14 (1.86)	2.53 (2.69)	0.389 (1.485)	ns
	Double dose	3.07 (3.03)	2.53 (3.43)	−0.535 (4.161)	ns
IL-6 (ng/L)	Control	2.63 (2.71)	1.81 (1.08)	−0.817 (2.329)	
	Single dose	2.29 (1.93)	2.16 (1.65)	−0.136 (1.210)	ns
	Double dose	2.97 (2.91)	2.58 (2.74)	−0.387 (2.679)	ns
15-keto-dihydro-$PGF_{2\alpha}$ (nmol/mmol creatinine)	Control	0.23 (0.07)	0.22 (0.06)	−0.014 (0.048)	
	Single dose	0.29 (0.14)	0.22 (0.07)	−0.077 (0.156)	ns
	Double dose	0.25 (0.05)	0.22 (0.05)	−0.026 (0.047)	ns

*Control group, $n = 13$, single dose group, $n = 13$, double dose group, $n = 14$.
†35–39 subjects.
‡38 subjects, CRP < 10 mg/L.

markers (CRP and IL-6), fasting glucose or insulin [48,49]. CRP levels decreased in patients supplemented with vitamin E (800 IU/day) but not in those treated with vitamin C (500 mg/day) or tomato juice (250 ml/day) in a 4-weeks intervention study. Fasting glucose did not change in any of the three treatment groups [50]. HbA1c, blood glucose and MDA were all reduced by a 2-month dietary treatment that increased the intake of vitamin C [51]. The protection against oxidative DNA damage, assessed by the comet assay, was increased by a 2-week dietary intervention including a flavonol rich diet [52]. The divergent outcomes in these interventional studies could be due to many factors, e.g. different types and doses of antioxidant supplements, the duration of the intervention, differences in the antioxidant targeted biomolecules or the status of glycaemic control. Baseline data from the present study showed positive associations between glycaemic control (blood glucose and HbA1c) and oxidative stress (8-iso-$PGF_{2\alpha}$ and nitrotyrosine) [53]. These associations are compatible with previous findings by Armstrong et al. [51], where dietary advice resulted in simultaneous decrease in oxidative stress (MDA) and improvement in glycaemic control (blood glucose and HbA1c). It indicates that improvement of glycaemic control may have a direct impact on oxidative stress that might mediate the effect of antioxidant supplementation on oxidative stress.

The observed increase in plasma levels of γ-tocopherol but not in α-tocopherol needs to be mentioned. In a previous study by Tomasch et al. [54], the effect of supplementation with two mixtures of α- and γ-tocopherol was investigated. A high content of γ-tocopherol compared to α-tocopherol increased the serum level of γ-tocopherol but not the serum level of α-tocopherol, a mixture composition and a result in accordance with the present study. A low content of γ-tocopherol compared to α-tocopherol increased the serum level of α-tocopherol but reduced the serum level of γ-tocopherol. These findings suggest that the balance between α- and γ-tocopherol in supplements may influence the serum levels.

The absence of an effect of antioxidant supplementation on glycaemic control, oxidative stress and inflammation in the present study might be explained by the population being studied. The concentrations of the investigated biomarkers of oxidative stress in our subjects were comparable with those observed in healthy subjects in other studies [3,12,41,45,55,56] with the exception of FPG-sites [55] and nitrotyrosine [7]. This is also true for the markers of inflammation [12,57], except for IL-6 [10]. These levels of oxidative stress and inflammatory biomarkers might have decreased the possibility to observe any effects of antioxidant supplementation. The levels of HbA1c in the present study indicate that the subjects in this study on average had relatively well-controlled diabetes, which might explain the low level of oxidative stress and inflammation. It is also possible that supplementation with antioxidants only is effective in decreasing oxidative stress and inflammation in subjects with a low plasma concentration of antioxidants and/or a low habitual intake of dietary antioxidants and fruit and vegetables. When investigating baseline values from this study, we have seen that there were negative correlations between the intake of dietary antioxidants and fruit and vegetables and biomarkers of both oxidative stress and inflammation [31]. The concentrations of plasma antioxidants in the present study were on average similar to those found in healthy subjects [58–61]. The dietary survey indicated that the subjects had an intake of dietary antioxidants and fruit and vegetables that was in accordance with the Swedish dietary recommendations [47]. The lack of a demonstrable effect might also be related to the choice of investigated markers of oxidative stress and inflammation. Since a relatively large number of biomarkers were included in the present study this explanation seems less probable.

Some limitations of this study deserve to be mentioned. The absence of a healthy reference group makes it difficult to confirm whether the subjects with diabetes in this study actually had increased levels of oxidative stress and inflammation or not. Comparisons with other studies have to be performed with caution. The absence of distinct criteria for inclusion of subjects that may benefit from antioxidant supplementation, e.g. defined reference values for biomarkers of oxidative stress, makes it difficult to design an optimal study of the effects of antioxidant intervention. The unique quality of the present study was the use of an innovatively composed antioxidant supplement containing a broad combination of antioxidants extracted from fruit, berries and vegetables to resemble the spectrum of antioxidants ingested via a daily intake of fruit and vegetables. Also, to our knowledge, no other study has previously investigated the effect of antioxidant supplementation in subjects with type 2 diabetes simultaneously performing this large number of complementary analyses of oxidative stress and inflammatory biomarkers.

In summary, we could not show that supplementation with a combination of antioxidants, extracted from fruit, berries and vegetables, improves the glycaemic control or lowers the level of oxidative stress or inflammatory biomarkers in a group of subjects with type 2 diabetes, despite markedly increased plasma antioxidant concentrations. The absence of an effect may be explained by the selection of the study group. It included subjects with relatively well-controlled diabetes, with a high intake of fruit and vegetables and with levels of plasma antioxidants, biomarkers of oxidative stress and inflammatory markers similar to those found in healthy subjects.

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