

Correlation between plasma levels of carotenoid and oxidized low density lipoproteins: A short human intervention study

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Abstract

The development of vegetable functional food products that provide benefits beyond their traditional nutritional value raised increasing interest. In fact several studies have shown that diet plays a role in the development of human diseases associated with oxidative damage (diabetes, cardiovascular disease and metabolic syndrome). Aim of the study was to evaluate the effects of daily intake of 300g of a frozen vegetable product containing red and green spinach, red and green chicory, red and green leaf chard (Italsur srl Notaresco, Italy) on plasma lipids and oxidized low density lipoproteins (ox-LDL). Furthermore, the bioavailability of lutein and β -carotene was investigated. The study included n=49 healthy volunteers (age ranged from 23 to 73 year), who consumed a portion of the vegetable product (300 g) every day for 2 weeks. The significant increase of plasma lutein and β -carotene after vegetable mix consumption for 2 weeks demonstrated that these phytonutrients are highly bioavailable. A significant reduction of plasma levels of total cholesterol (TC) and LDL-cholesterol (LDL-C) was observed after dietary intervention. The results demonstrated a significant decrease in plasma concentration of ox-LDL after treatment. Evaluation of ox-LDL represents a useful biochemical marker of lipid peroxidation; therefore the results demonstrated a decrease of lipid peroxidation of lipoproteins associated with the consumption of vegetable product. A significant negative correlation has been established between levels of plasma lutein and levels of ox-LDL before and after daily intake of the vegetable product. These results suggest that lutein may play a role in the protective effect against oxidation of LDL. In conclusion the increase of plasma carotenoids after dietary treatment for two weeks is associated with a protective effect against lipid peroxidation of lipoproteins.

Introduction

The frozen ready-to-eat vegetables market has been growing and deserves increasing attention due to modifications of lifestyle and changes of dietary habits. Accurate selection of vegetables can result in convenient, healthy and appealing products, which may contain interesting bioactive compounds. Among vegetable foods that could be defined as functional foods there are pigmented vegetables such red spinach, green spinach, red chicory and red chard. Previous studies have demonstrated that they contain bioactive molecules and phytochemicals (polyphenols and carotenoids), vitamins (vitamin C, folate, and provitamin A), minerals (potassium, calcium, and magnesium) and fibers [1-5]. However their biological effects have been mainly investigated *in vitro* and in model animals [6-10].

Among phytonutrients, carotenoids have been widely investigated for their protective role against lipid peroxidation. In fact plasma carotenoid levels are associated with low density lipoproteins in particular [11]. The correlation between dietary intake of carotenoids and plasma carotenoid levels has been previously demonstrated [12-18]. Therefore the increase in plasma level of carotenoids after dietary intake of vegetable has been suggested as a useful nutritional marker of their bioavailability in human subjects. An increased plasma total antioxidant capacity has also been associated with a consumption of vegetable antioxidants [13,19,20]. The bioavailability of bioactive nutrients is modulated by different factors such as chemical composition of vegetable and processing [19,21,22].

In order to evaluate the nutritional properties of a ready to eat frozen product and the bioavailability of the main bioactive molecules, we investigated the plasma levels of carotenoids and antioxidant potential after intake of a experimental ready-to-eat product containing pigmented vegetables that are rich sources of polyphenols, carotenoids and other phytochemicals. Therefore we enrolled 48 volunteers whose diet was supplemented for two weeks with a daily portion of the vegetable product. Moreover we studied the effect of the intake of the product on the markers of cardiovascular disease such as plasma lipid profile and levels of oxidized LDL.

Materials and methods

Subjects

The study was conducted during September–November 2012. The inclusion criteria for subjects were: not taking vitamins, minerals,

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or other types of supplements during the previous two months; no-smoking; BMI within the normal range according to the World Health Organization criteria (18.5–25 kg/m²) and normal biochemical and haematological profile (serum cholesterol <6.8 mmol/L, serum triacylglycerols <2.8 mmol/L, glucose <6.11 mmol/L).

The exclusion criteria were: diagnosed diseases such as allergies, cancer, diabetes, obesity, hypertension, mental diseases, gastrointestinal or renal diseases, as well as intake of drugs related to these pathologies, alcohol consumption >30 g/day, vegetarian diet. None of the female subjects was pregnant or lactating. Volunteers were recruited in the Polytechnic University of Marche (UNIVPM), Italy.

Forty-eight subjects followed the diet, 22 of them (45.8%) were males. The median age was 40.5 years (1st-3rd quartile: 36 – 47 years) and median BMI 23.6 kg/m² (1st-3rd quartile: 21.8 – 26.4 kg/m²). The mean plasma lipid measurements for the subjects are summarized in Table 1.

Intervention study

The intervention phase consisted of a 2-week period which included daily consumption of a portion (300 g) of the vegetable frozen product supplied by Italsur srl (Notaresco, Teramo, Italy). The vegetable frozen product contains the same proportions of red spinach (*Spinacia oleracea*), green spinach (*Spinacia oleracea*), red chicory and green chicory and a lower percentage of green chard (*Beta vulgaris L. var. cicla*) and red chard (*Beta vulgaris L. var. cicla*). Macronutrients contained in a portion of the final product were evaluated [23]. Water-soluble vitamins were quantified by high-performance liquid chromatography/electrospray ionization-mass spectrometry [24]. Carotenoids were analyzed using high performance liquid chromatography (HPLC) [25]. Total polyphenols were evaluated following Xu *et al.* [26]. Total antioxidant potential was evaluated by ORAC assay [4]. Table 2 shows the percentage of the energy, macro and micronutrients found in the vegetable product.

The product was consumed after cooking for 15 minutes with 2 tablespoons extra virgin olive oil. Intake of the vegetable product was included in the normal daily diet and no specific time of consumption or accompanying meal was established. Subjects were recommended to maintain their habitual dietary intake (especially as regard to their consumption of a provided list of foods with high carotenoid, polyphenol and vitamin C contents) and their usual physical activity or other lifestyle habits. Moreover, they were requested to record any sign of illnesses, medications, and any deviations from their experimental diets. No subject was reported to have side effects and thus, no one

Table 1. Plasma lipid profile, levels of ox-LDL and total antioxidant capacity subjects in plasma of LDL in plasma of subjects at baseline and after 2week period which include a daily portion of the ready-to-eat product containing pigmented vegetables. (Data are shown as mean± standard deviation). A $p < 0.05$ was considered statistically significant.

	Baseline (n=49)	After 2-weeks (n=49)	P value
TC (mmol/L)	4.87 ± 0.56	4.55 ± 0.52	$p < 0.001$
LDL-C (mmol/L)	2.80 ± 0.56	2.53 ± 0.52	$p < 0.001$
HDL-C (mmol/L)	1.65 ± 0.28	1.65 ± 0.30	$p = 0.5$
TG (mmol/L)	0.91 ± 0.41	0.87 ± 0.37	$p = 0.06$
Fasting glucose (mmol/L)	4.88 ± 0.43	4.83 ± 0.31	$p = 0.07$
Total antioxidant capacity (mmol TE/L)	14634 ± 3492	16218 ± 3834	$p < 0.001$
ox-LDL (U/L)	41.7 ± 16.1	32.6 ± 12.2	$p < 0.001$
ox-LDL/LDL-C (U/mmol)	15.2 ± 5.6	13.2 ± 5.2	$p < 0.001$

Table 2. Macronutrients, vitamins and phytochemicals in a portion (300 g) of vegetable product containing red and green spinach, red and green chicory, red and green leaf chard (Italsur srl Notaresco, Italy). (Data are reported as mean ± standard deviation of data from analyses carried out in samples from different batch and different years (2011 and 2013)).

	Composition of a portion of vegetable product (300 g)
Energy (kcal)	18 ± 2
Protein (g)	7.8 ± 1.1
Carbohydrate(g)	11.0 ± 2.2
Fiber(g)	8.5 ± 0.18
Fat(g)	0.63 ± 0.01
Saturated fatty acids(g)	0.15 ± 0.01
Monounsaturated fatty acids (g)	0.06 ± 0.01
Vitamin C (mg)	85 ± 3.7
Folate(mg)	0.33 ± 2.7
Alpha-tocopherol(mg)	3.9 ± 0.30
Niacin(mg)	1.2 ± 0.05
Retinol(mg)	3.2 ± 0.20
Riboflavin(mg)	0.27 ± 0.03
Thiamine(mg)	0.18 ± 0.01
Total Polyphenols	530 ± 82
Caffeic acid (mg)	80.1 ± 3.7
Chlorogenic acid (mg)	18.2 ± 1.5
Coumaric acid (mg)	3.4 ± 0.34
Ferulic acid (mg)	2.3 ± 0.11
Betacyanin (mg)	26.7 ± 1.5
Luteolin (mg)	4.7 ± 0.9
Kaempferol (mg)	1.23 ± 0.17
Quercetin (mg)	1.91 ± 0.11
Isorhamnetin (mg)	1.32 ± 0.02
β-carotene (mg)	11.1 ± 0.8
Zeaxanthin (mg)	0.7 ± 0.1
Lutein (mg)	29.6 ± 0.30
Total ORAC (mmol(TE))	5.8 ± 0.1

dropped out during the experimental period. At the beginning of the intervention (baseline, T0) and at the end (after 2 weeks, T15), fasting blood samples were collected. The study was performed in accordance with the Helsinki Declaration of Human Studies and approved by the Ethical Committee of the “Azienda Ospedaliero-Universitaria Ospedali Riuniti” Ancona (Italy) (Protocol number 211525). All participants signed an informed consent document.

Analytical determinations

At the beginning of the study and after two weeks of dietary treatment, fasting blood samples (10 mL) were collected from each subject by venipuncture from the antecubital vein: 5 mL were placed in heparin tubes for haematological measurements, while 5 mL were placed in tubes without any anticoagulant and centrifuged at 1500 g for 10 min at 4°C for serum separation. Plasma and serum aliquots were prepared and stored at –80°C until analysis. Analyses did not commence until the full intervention study was completed and all samples from each subject were analysed within one batch to reduce interbatch variation.

Plasma lipids and glycaemia

Serum glucose, triacylglycerols (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) were analyzed by commercial kits (Chemadiagnostica, Jesi, Italy). Low density lipoprotein cholesterol (LDL-C) was calculated by the Friedewald formula.

Plasma carotenoids

Carotenoids (β -carotene and lutein) were quantified in plasma of subjects by high-performance liquid chromatography (HPLC) system, using a single dilution step after extraction with propanol (1:5, v/v) and vigorous vortexing of 250 μ L of extraction mixture. This mix was centrifuged for 2 min at 20,000 g at 4°C. Forty microlitres of super natant were injected into the HPLC with electrochemical detector (ECD) by Shiseido (Tokyo, Japan), using a pre-separation concentrating column 50 \times 2.0 mm ID 5 μ m, separation C18 column 150 \times 2.0 mm ID 3 μ m, and a post-separation reducing column CQR 20 \times 2.0 mm, all from Shiseido. For each carotenoid quantified, two mobile phases were used. Mobile phase 1 for loading and concentrating the sample (50 mM sodium perchlorate in methanol/water 95:5, v/v) was the same for both molecules, while mobile phase 2 was 50 mM sodium perchlorate in methanol/isopropanol (80:20, v/v) for lutein and 50 mM sodium perchlorate in methanol/isopropanol (98:2, v/v) for β -carotene. Moreover, flow rate was 200 μ L/min for phase 1 in both analyses. Flow rates for phase 2 were 300 and 80 μ L/min for β -carotene and lutein, respectively. Total chromatographic run times and retention times were 24 min/12.3 min for β carotene and 21 min/9.8 min for lutein [12].

Plasma total antioxidant capacity (PAT)

Plasma total antioxidant capacity (PAT) was measured using oxygen radical absorbance capacity (ORAC) adapted for semi-automated measurement on a 96-well microplate reader (Synergy HT; BioTek, Winooski, VT, USA) [27].

Markers of lipid peroxidation

In-vivo oxidized LDL were determined in plasma by a sandwich ELISA procedure using the murine monoclonal antibody mAB-4E6 as the capture antibody, and a peroxidase conjugated antibody against oxidized apolipoprotein B bound to the solid phase (ox-LDL, Mercodia AB, Uppsala, Sweden). Intra and inter-assay CVs were 2.82% and 7.29%, respectively. As LDL-C is considered a major determinant of absolute ox-LDL levels, plasma values of ox-LDL (U/L) were adjusted by the plasma levels of LDL-C (mmol/L) by calculating their ratio (units of ox-LDL per mmol of LDL-C), in agreement with Zuliani *et al.* [28].

Statistical analysis

Mean value, standard deviation (DS), and mean standard error (SEM) were calculated. By using Student T test, we evaluated the significance of differences between mean values at study entry and following treatment. A p of 0.05 was considered statistically significant. Pearson correlation coefficients and their significance levels were calculated for linear regression analysis.

Results

Macronutrients and micronutrients contained in the frozen ready-to-eat product containing pigmented vegetables are summarized in Table 2. A portion of the product (300g) contains 8.5 ± 0.18 g and provides 34% of recommended daily intake of fiber (25 g/day). It contains high levels of vitamin C (85 ± 3.7 mg) and folate (0.33 ± 2.7 mg); a portion provides more than 100% of recommended dietary allowances (RDA) of both vitamins (Commission Directive 2008/100/EC). The levels of carotenoids were 11.1 ± 0.8 mg of β -carotene and 29.6 ± 0.30 mg of lutein (Table 2). As shown in Table 2, among polyphenols, phenolics acids and flavonols are the main components in the vegetable product. Anthocyanins and flavones have also been

identified (Table 2). Using ORAC assay to evaluate total antioxidant capacity, the product ranks highest among vegetables [29]. In fact a portion of the frozen product provides 5.8 ± 0.1 mmol/TE.

Plasma lipids

Subjects at baseline were characterized by normal levels of the lipid profile (Table 1). A significant decrease occurred in TC, LDL-c after 15 days of diet are presented in Table 1. No significant difference was found in the absolute variation of HDL cholesterol levels and triacylglycerol levels (Table 1).

Carotenoids

Basal levels of lutein ranged from 0.16 μ g/mL to 0.75 μ g/mL and basal levels of β -carotene ranged from 0.07 μ g/mL to 0.53 μ g/mL, the median values are in agreement with previous studies by us and other authors (Figure 1) [13,30].

Figure 1 shows the variation in plasma concentrations of lutein and β -carotene after daily intake of the experimental product compared with baseline. A significant increase of plasma carotenoids (lutein and beta-carotene) was found after 15 days of supplemented diet ($p < 0.001$).

Levels of oxidized LDL and total antioxidant properties

Plasma levels of ox-LDL were determined as biomarkers of lipid peroxidation during the study. The basal levels of ox-LDL were similar to those reported in previous studies by us and other studies on healthy subjects (Table 1) [13, 28]. Significant decrease of the plasma levels ox-LDL and ox-LDL/LDL ratio was found after 15 days of supplemented diet (Table 1).

Relationship between plasma carotenoids and markers of lipid peroxidation

As shown in Figure 2 a significant negative correlation has been established between levels of plasma lutein and levels of ox-LDL ($r = 0.59$, $n = 98$, $p < 0.001$) and ox-LDL/LDL-C ($r = 0.61$, $n = 98$, $p < 0.001$) before and after daily intake of "Mix of chard, chicory and spinach"; no significant correlations were observed with plasma levels of β -carotene and levels of markers of lipid peroxidation.

Discussion

The phytochemical composition of pigmented vegetables has been

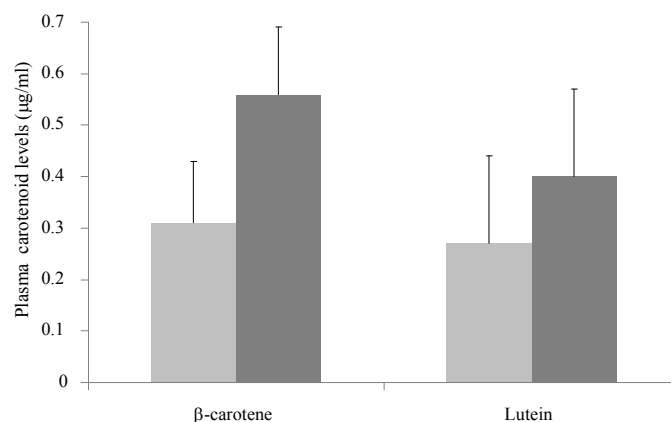


Figure 1. Levels of carotenoids (beta carotene and lutein) in plasma of subjects at baseline (◇) and after 2 weeks period (◆) which include a daily portion of the ready-to-eat product containing pigmented vegetables.

* $p < 0.001$ vs. β -carotene levels in subjects at baseline; ** $p < 0.001$ vs. lutein levels in subjects at baseline.

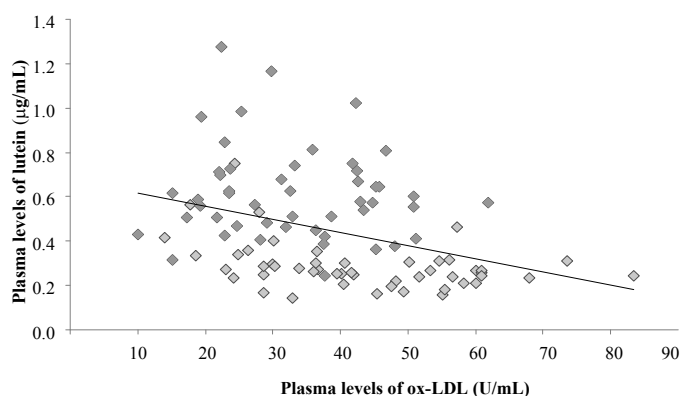


Figure 2. Correlation between levels of lutein and of oxidized LDL in plasma of subjects at baseline (◆) and after 2 weeks period which included a daily portion of the ready-to-eat product containing pigmented vegetables (◆).

previously investigated [1-5]. However the biological effect on human subjects has not been previously studied.

A portion of the vegetable product contains about 30 mg lutein and 11 mg beta carotene. Serum carotenoid concentration increased after 2 weeks, beta-carotene concentration by 56% and lutein concentration by 94.5% compared with baseline. These data demonstrated that these phytonutrients in the product are bioavailable. In our previous study we have demonstrated that the daily intake for two weeks of a portion of the vegetable combination of black and red cabbage containing 1 mg lutein and 0.6 mg β -carotene, increased plasma β -carotene and lutein concentration by 80 and 204%, respectively compared with the baseline [13]. Other authors have demonstrated that three weeks of intake of 9.3 mg of beta-carotene from spinach products increased serum concentration by 53% [31]. Martinez-Thomas *et al.* [32] have confirmed an increase of beta-carotene by 114% after three weeks 3.9 mg of beta-carotene contained in a fruit and vegetable soup. The inter-relationship of different carotenoids present in food matrix affects carotenoid absorption and could explain the different percentage increase. In particular it is well known that lutein modulates beta-carotene bioavailability [33].

Using the evaluation of the total antioxidant capacity, that represents a suitable biochemical parameter for assessing the overall antioxidant status, we demonstrated a significant increase. Our results demonstrated also that the daily consumption of the product for 2 weeks is associated with a significant reduction of total-cholesterol and LDL-C in absence of changes of HDL-C and TG levels.

A portion of the vegetable product contains 8.5 g of fibers and about a third (2.5 g) is represented by soluble fiber. Extensive research has shown that fibres play an important role in cholesterol metabolism by decreasing plasma TC and LDL-C, as demonstrated in a meta-analysis [34]. In general, most soluble fibers lower plasma total cholesterol more efficiently than water-insoluble fibers by decreasing LDL cholesterol without significantly affecting the HDL-C and TG levels [34]. Other phytochemicals contained in the product such as polyphenols could contribute to the LDL cholesterol lowering effect. This hypothesis is supported by previous studies, *in-vivo*, which has demonstrated that diets naturally rich in polyphenols improve plasma lipid profile [35-37]. Moreover, *in vitro* studies, have shown that individual polyphenols such anthocyanins, quercetin, kaempferol and luteolin are able to modulate cholesterol absorption [38,39] and lipid synthesis [40].

The decrease of oxidized LDL could be related to the modifications

of carotenoid and/or other phytochemicals as suggested by previous human studies [12-17]. By the product analysis it was evidenced a high content of vitamin C. Previous studies have observed an inverse correlation between vitamin C concentrations and 8-epi-PGF2 concentrations in subjects at baseline and after 2 week of supplementation with the vegetable soup [41,42], moreover there is convincing evidence that vitamin C is a strong inhibitor of LDL oxidation [43], a recognized factor in the pathogenesis and progression of human atherosclerosis, by scavenging free radicals and other reactive species, and preventing their interaction to oxidize LDL [43]. All the aforementioned nutrients may have synergistically contributed to the protective effect associated with consumption of the vegetable product.

The statistical analysis of the effect of dietary intervention on biochemical parameters has demonstrated variability. A significant higher increase of lutein and β -carotene levels was observed in females. Moreover, subjects with values of TC, HDL-c, triacylglycerol, ox-LDL and ox-LDL/LDL ratio, over the median, had a higher decrease of the dependent variable. This variability in response must be associated with genetic or other characteristics of the individual [44].

Diet is considered the cornerstone for the prevention of age-related diseases, and a low-fat diet has been considered for decades as the most suitable alternative to achieve this goal. However, mounting evidence supports the efficacy of other alternatives, such as the Mediterranean diet and intake of foods rich in antioxidants and phytochemicals [45].

In conclusion the synergistic effects of phytochemicals (dietary fibers, polyphenols, carotenoids) present in the frozen ready to eat product could be responsible for the protective effects and benefits observed could be attributed to the complex mixture of phytochemicals present in whole foods. High levels of LDL-cholesterol and of oxidized LDL are biochemical markers for atherosclerosis [46,47]. Therefore we suggest that products rich in carotenoids and other phytochemicals could be inserted in dietary intervention aimed to prevent cardiovascular diseases and their complications.

Despite dietary health effects are influenced by genetic factors [44], an accurate selection of pigmented vegetable results in palatable, convenient and healthy product with physiological effects on plasma lipids and lipid peroxidation.

Authors' contribution

All authors contributed to the intellectual development of this work and approved the final manuscript. TB and GF were responsible for the experimental design, coordination of research and preparation of the manuscript; TB and SM carried out evaluations of plasma biochemical parameters (plasma lipids, markers of lipid peroxidation) and participated in the preparation of the manuscript; D.T. was actively involved in the chemical and agronomic investigation of plant species used to make the frozen product; LT, FB, SS and PO carried out evaluations of plasma carotenoids and contributed to the preparation of the manuscript.

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