Eur J Nutr 41 : 95-100 (2002)

ORIGINAL CONTRIBUTION

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Spinach and tomato consumption increases lymphocyte DNA resistance to oxidative stress but this is not related to cell carotenoid concentrations

Summary *Background* The increased consumption of fruit and vegetables has been linked to protection against different chronic diseases, but the dietary constituents responsible for this association have not been clearly identified. *Aim of the study* We evaluated the effect of spinach and spinach+tomato puree consumption on cell DNA resistance to an oxidative stress. *Methods* To this aim, in a dietary controlled intervention study, 9 healthy female volunteers consumed a basal diet low in carotenoids (< 600 µg/day) enriched with daily portions (150 g) of spinach (providing about 9 mg lutein, 0.6 mg zeaxanthin, 4 mg β-carotene) for 3 weeks (from day 0 to day 21) followed by a 2 week wash-out period (basal diet) and finally another 3 weeks (from day 35 to day 56) of diet enriched with daily portions of spinach (150 g) + tomato puree (25 g, providing about 7 mg lycopene, 0.3 mg β-carotene). At the beginning and the end of each period of vegetable intake, blood samples were collected for lymphocyte separation. Carotenoid concentrations of lymphocytes were determined by HPLC and DNA damage was evaluated by the comet assay following an *ex vivo* treatment with H_2O_2. *Results* During the first period of spinach consumption, lymphocyte lutein concentration did not increase significantly (from 1.6 to 2.2 µmol/10^{12} cells) while lycopene and β-carotene concentrations decreased significantly (from 1.0 to 0.1 µmol/10^{12} cells, $P < 0.001$, and from 2.2 to 1.2 µmol/10^{12} cells, $P < 0.05$, respectively). Lutein and lycopene concentrations increased after spinach+tomato puree consumption (from 1.2 to 3.5 µmol/10^{12} cells, $P < 0.01$, and from 0.1 to 0.7 µmol/10^{12} cells, $P < 0.05$, respectively). The increase may be attributed to the addition of tomato puree to spinach; however, the different concentrations of carotenoids in lymphocytes registered at the beginning of the two intervention periods may have affected the results. DNA resistance to H_2O_2 insult increased significantly after both the enriched diets ($P < 0.01$); however, no "additive effect" was seen after spinach + tomato puree consumption. In the spinach + tomato intervention period an inverse correlation was observed between lymphocyte lycopene concentration and DNA damage, but this seems not able to explain the protection observed. *Conclusions* The consumption of carotenoid-rich foods even for a short period of time gives protection against oxidative stress. The results obtained seem to suggest that this protective role is not specifically related to carotenoids. However they may contribute together with other substances present in vegetables to lymphocyte resistance to oxidative damage.

Key words Vegetables – carotenoids – DNA damage – lymphocytes

Received: 20 September 2001
Accepted: 18 February 2002

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EJN 359

European Journal of Nutrition, Vol. 41, Number 3 (2002)

Introduction

Fruit and vegetables have been linked with a protective action against different chronic diseases [1–4]. There are many dietary constituents that could be responsible for this association and different studies have been carried out to try to identify the most effective compounds. Among these compounds, carotenoids would seem to contribute to the beneficial effects of fruit and vegetable consumption [5, 6], but so could many other substances, such as vitamin C, folates, flavonoids, thus, making it difficult to come to conclusive results about the effect of each single compound. For this reason, and considering the inconclusive results of intervention trials with pure substances, e. g., the Alpha-Tocopherol Beta-Carotene ATBC [7] and the Carotenoid Retinol (CARET) [8] trials, it would be more sensible to study how an increased amount of foods rich in these protective compounds could influence good health. In fact, there are not many controlled dietary intervention studies in this regard [9]. Cao et al. [10] demonstrated that increased consumption of fruit and vegetables can improve the plasma antioxidant capacity, measured as oxygen radical absorbance capacity (ORAC). Hininger et al. [11] showed that an increased intake of fruits and vegetables rich in carotenoids for two weeks enhanced the resistance of LDL to oxidation by 14 % in smokers (11 subjects) and 28 % in non-smokers (11 subjects). Pool-Zobel et al. [12] found a decrease in the endogenous levels of strand breaks in lymphocyte DNA after the consumption of tomato juice, but also of carrot juice and dried spinach. However, by considering the experimental design (without a wash-out period) the authors could not exclude possible delayed effects of earlier supplementation (e. g., tomato juice) on subsequent treatments (e. g., carrots and spinach). We provided further evidence of the protective effect of tomato consumption on lymphocyte DNA resistance to an oxidative stress using the comet assay [13, 14].

In the present work we studied the effect of spinach consumption on cell DNA resistance in relation to carotenoid bioavailability to cells. Spinach is rich in lutein, one of the main carotenoids found in human plasma, which exerts several important biological functions such as the protection from age-related macular degeneration [15, 16], the enhancement of immune function [17] and the reduction of cancer development [18, 19]. Most of these protective effects of lutein are exerted through its antioxidant activity which has been evaluated by *in vitro* [20] and *ex vivo* models [21]. However few studies report the effect of consumption of vegetables rich in lutein on biomarkers of the oxidative stress [22]. Furthermore no data are available on the effects of the consumption of different vegetables together; consequently the potential interaction between spinach and tomato was considered.

Subjects and methods

Subjects

The experimental procedure was performed on nine non-smoking healthy female subjects with no history of cardiovascular, hepatic, renal, or gastrointestinal disease, with a mean (± SD) age of 25.2 ± 2.2 y and a mean body mass index (BMI; kg/m^2) of 20.2 ± 1.6. They were selected on the basis of their eating habits in order to have an homogeneous group for eating behavior. Vegetarians and subjects on specific diet or regimen were excluded.

Informal written consent was obtained from each participant and the protocol was in accord with ethical standards of the Local Ethics committee.

Experimental design

Subjects were asked to consume a diet enriched with daily portions of spinach for 3 wks (from day 0 to day 21) followed by a 2 wk wash-out period and finally another 3 wks (from day 35 to day 56) of diet enriched with daily portions of spinach + tomato puree.

One week before the beginning of the study and during all the period of experimentation (56 days), subjects followed a basal diet in order to limit carotenoid intake (< 600 µg/day) and avoid lutein and lycopene without interfering with their own eating habits. They were provided with a list of allowed and not allowed foods and were asked to follow a detailed menu regarding the type and amount of fruit and vegetables to eat at each meal. This procedure was chosen to limit possible differences in intakes due to seasonal variation.

For the study, subjects received 150 g of spinach (chopped and frozen, Bonduelle Italia, Brescia, Italy) providing about 9 mg lutein, 0.6 mg zeaxanthin and 4 mg β-carotene. In the second part of the experiment 25 g tomato puree (double-concentrate tomato puree; Sainsbury's, London, UK) was added to the spinach providing further 7 mg lycopene and 0.3 mg β-carotene. The spinach was consumed after microwave heating for 5 min at moderate power while the tomato puree was eaten uncooked. The foods were eaten with 10 g olive oil at dinner. Compliance with the diet was assessed by a dietician.

Blood samples

Blood samples for lymphocyte separation were collected at the beginning and the end of each period of supplementation (day 0, 21, 35, 56) early in the morning after overnight fasting.

To separate lymphocytes, 10 mL of whole blood was

centrifuged (400 × g, 30 min) and recovered by means of a density gradient separation with Histopaque 1077 (Sigma Chemical Co, St Louis). The lymphocyte layer was withdrawn, the cells washed with PBS and counted using a hemocytometer.

Extraction of carotenoids

Food samples. The extraction was performed according to a method previously reported [23] that consists of a first step of exhaustive extraction by means of THF and methanol followed by a further step of separation using petroleum ether and water. The final sample, dissolved in the HPLC mobile phase, was injected in the chromatographic system to quantify the carotenoid content [23].

Lymphocyte. Carotenoid extraction from lymphocytes was performed after the treatment of the cells with triton X 100 (Sigma) (1 % in PBS) and subsequent lysis by means of liquid nitrogen freezing and thawing. Two milliliters ethanol (containing echinenone as the internal standard) and 2 mL hexane were added to the final sample. After vortexing for 1 minute the organic layer was separated. A subsequent extraction with 2 mL hexane was performed and the organic layer was separated and added to the previous one. The sample was then dried under N_2 and solubilized in 100 µL of the HPLC mobile phase for the carotenoid analysis.

HPLC analysis of carotenoids

Carotenoid HPLC analysis was performed as previously described [24] by using a 5 µm Vydac 201 TP 54 C_{18} column (250 × 4.6 mm, i. d.) fitted with a C_{18} guard column and biocompatible frits. The eluant was methanol:THF (95:5) at a flow rate of 1 mL/min. Visible detection was achieved at 445 nm (UV-VIS detector Varian 2010). Recovery was between 90 % and 100 %.

Carotenoid concentrations were calculated by means of a mix of standards containing lutein, zeaxanthin, β-cryptoxanthin (Hoffman-La Roche, Basel, Switzerland), α-carotene and β-carotene (Sigma) while lycopene (Sigma) was prepared daily to avoid problems of degradation, and injected separately. Data were then corrected by the recovery of the internal standard.

DNA damage of lymphocytes

All procedures and the Comet assay used to evaluate DNA damage were performed as previously reported in detail [13]. The lymphocytes were separated by density gradient from 70 µL of whole blood. The cells recovered were resuspended in about 80 µL PBS.

Two samples for each subject were prepared. One sample in agarose placed on a fully frosted microscope slide (Richardson Supply Co. London, United Kingdom) was subjected to a H_2O_2 treatment to study the resistance of lymphocytes to oxidative stress while the other sample acted as the control. The treatment consisted of the slides being placed for 5 minutes in a solution of H_2O_2 in PBS (500 µmol/L). The slides were then put in cold lysis buffer and kept at 4 °C for 1 hour in the dark. Subsequently the slides were left at 4 °C for 40 minutes in fresh electrophoresis buffer in a horizontal electrophoresis tank (Scotlab, Coatbridge, United Kingdom), prior to electrophoresis in the same solution at 25 V, 300 mA for 20 minutes at 4 °C in the dark. After neutralization of the alkali and detergents, the slides were stained with ethidium bromide in neutralizing buffer (2 µg/mL) and washed in PBS, drained and covered with coverslips.

An epifluorescence microscope (BX60; Olympus Italia, Milan, Italy) attached to a high sensitivity CCD video-camera (Variocam; PCO Computer Optics, Kelheim, Germany) and to a computer provided with an image analysis system was used to check the slides. Fifty cells for each slide were electronically captured and analyzed for fluorescence intensity. The damaged DNA is recognized as a fluorescent core followed by a tail due to the presence of strand breaks in the chain. DNA damage was calculated as % DNA in tail. For each subject the mean % DNA in tail of treated cells was subtracted from the % DNA in tail of control cells.

Statistical analysis

Statistical analyses were performed on a personal computer with STATISTICA software (Statsoft Inc, Tulsa, OK).

A repeated-measures analysis of variance (ANOVA) with type of treatment and time as dependent factors was used to investigate the effect of spinach or spinach + tomato puree consumption on carotenoid lymphocyte concentrations and on lymphocyte DNA damage. Differences were considered significant if $P < 0.05$.

The analysis of simple regression was used to evaluate the correlation between variables (lymphocyte carotenoid concentration vs. DNA in tail).

Results

Lymphocyte concentration of carotenoids

The carotenoid concentrations of lymphocytes before and after spinach and spinach + tomato puree consumption are reported in Table 1. There was an increase in lymphocyte lutein concentration after both supple-

mentations; however, this was significant only after spinach + tomato puree consumption ($P < 0.01$). β-carotene showed a decrease after spinach and an increase after spinach + tomato puree consumption. Lycopene concentration decreased significantly during the spinach consumption ($P < 0.001$) and increased after spinach + tomato puree consumption ($P < 0.01$). The other carotenoids did not vary significantly.

From these data we observed that the intake of spinach and tomato puree together caused an increase of all the three main carotenoids. The highest increase was registered for lycopene, 7 times the basal value (day 35), while lutein increased by 3 times and β-carotene was only double the initial concentration.

DNA damage

Both periods of supplementation significantly affected the resistance of lymphocytes from the oxidative insult (Fig. 1). DNA damage significantly decreased after the enriched diets ($P < 0.01$).

No relation was observed between lutein lymphocyte concentration and DNA damage, while this was inversely correlated to lycopene concentration after spinach + tomato puree consumption ($R = -0.6$, $P < 0.001$).

Table 1 Lymphocyte carotenoid concentrations before and after spinach and spinach + tomato puree consumption[a]

	Spinach		Spinach + tomato puree	
	day 0	day 21	day 35	day 56
	μmol/10^{12} cells			
Lutein	1.6±0.2	2.2±0.3	1.2±0.3	3.5±0.5[c]
Zeaxanthyn	0.2±0.02	0.1±0.07	0.04±0.02	0.1±0.02
β-cryptoxanthyn	2.4±0.7	0.9±0.2	0.6±0.08	0.8±0.1
α-carotene	0.6±0.2	0.3±0.04	0.06±0.03	0.1±0.05
β-carotene	2.2±0.4	1.2±0.3[e]	1.0±0.2	2.1±0.4[f]
Lycopene	1.0±0.2	0.09±0.05[b]	0.1±0.03	0.7±0.1[d]

[a] mean ± SE
[b] Significantly different from day 0, $P < 0.001$
[c, d] Significantly different from day 35, $P < 0.01$
[e, f] Significantly different from day 0 and day 35 respectively, $P < 0.05$

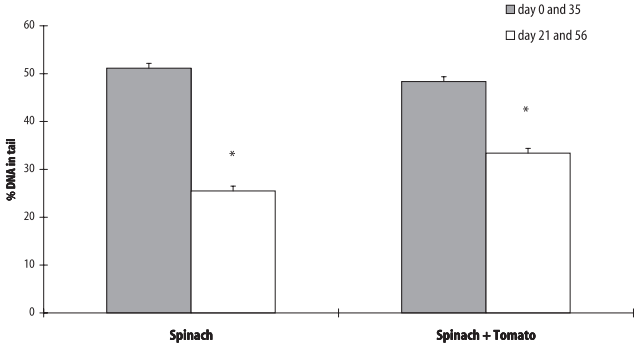

Fig. 1 Lymphocyte DNA damage (mean ± SE) as evaluated by the Comet assay before and after spinach and spinach + tomato puree consumption. * Significantly different from day 0, and day 35 respectively $P < 0.01$.

Discussion

Spinach and tomato are widely consumed by mediterranean populations and are sources of important carotenoids such as lutein, lycopene, and β-carotene. These carotenoids are recognized as powerful antioxidants and it is reported they could exert a specific action, in consideration of the different accumulation in human tissues [25]. We previously demonstrated that the daily consumption of tomato puree was able to improve lymphocyte resistance to an oxidative stress induced *ex vivo* by H_2O_2 [13, 14].

In the present study we investigated whether this protective effect could also be exerted by spinach and whether consuming spinach and tomato together could provide even better protection.

To discuss our data, it is important to remember the study design adopted, which involved 1 preliminary week of basal diet low in carotenoids followed by 3 weeks of spinach consumption, a 2 week wash-out (just the basal diet) and a further 3 weeks of spinach + tomato puree consumption. Following this design, lutein concentration increased by about 37% after spinach consumption (day 21) but this increase was not significant, then it decreased during the wash-out period (day 35) and tripled when spinach + tomato puree were consumed (day 56). Lycopene concentration decreased during spinach consumption and remained constant and in a negligible amount during the wash-out. The intake of tomato together with spinach significantly increased lycopene concentrations (about 7 times the initial value), however without reaching the basal level (day 0).

The capability of lymphocytes to protect themselves against H_2O_2 insult increased significantly after consuming both spinach and spinach + tomato puree for 3 weeks. However the combination of tomato puree with spinach had no "additive effect" on the degree of protection. It is interesting to underline that the degree of protection reached in both intervention periods is comparable to that previously observed, when subjects were given only tomato puree: 60 g for 3 weeks (16.5 mg lycopene and 0.3 mg β-carotene) [13] or 25 g for 2 weeks (7 mg lycopene and 0.3 mg β-carotene) [14].

Also Pool-Zobel et al. [12] found a significant decrease in endogenous DNA damage, evaluated by the comet assay, following the consumption of 10 g dried spinach (11.3 mg lutein) or 300 ml tomato juice (40 mg lycopene) for two weeks, and a decrease in oxidized pyrimidine after carrot juice supplementation (about 22 mg β-carotene and 15 mg α-carotene).

From the data available, it could be presumed that in general, vegetables rich in carotenoids can improve the antioxidant capacity of the cells, but whether these effects are related to carotenoid activity has to be clarified.

In the present study we did not find any significant correlation between lutein concentration and DNA damage, even though the first increased and the latter decreased in both intervention periods. This could depend on the high variability in lutein concentration between subjects and/or the fact that the relation between antioxidant concentrations and their potential protective effect cannot be described with simple or linear models. In contrast, in our previous study [14] we found that lycopene concentration was inversely associated with DNA damage in lymphocytes of volunteers consuming tomato puree. In the present experiment, however, lycopene concentration was too low (due to the 3 plus 2 wk period of lycopene-free diet) to suggest its relevant contribution to DNA protection.

Borel et al. [26] reported that the oxidative stress status (evaluated by means of breath pentane measurement) and the antioxidant status (evaluated by the total antioxidant capacity of plasma) of a group of healthy subjects was apparently not related to the carotenoid concentrations found in plasma and tissues. They suggested that high oxidative stress may be required to find a significant effect of carotenoids on breath pentane output or that alternatively carotenoids do not play a major role in the antioxidant defence against peroxidation. Also Collins et al. [27] came to similar conclusions in an intervention study in which subjects supplemented with α-carotene, β-carotene, lutein, lycopene and placebo did not show any difference in lymphocyte endogenous DNA oxidative damage. Moreover recently Torbergsen and Collins [28] found an apparent enhancement of DNA repair in lymphocytes after carotenoid supplementation (particularly lycopene and β-carotene); however, the authors suggested this was due to an antioxidant effect against additional DNA damage induced by atmospheric oxygen rather than a stimulation of DNA repair.

In our study, the reduction of DNA damage in lymphocytes subjected to oxidative stress may support just a contribution of lutein, lycopene and the other carotenoids to the antioxidant defence system of the cells or, alternatively, may suggest that carotenoids could be nothing more than a marker of "increased protection of the cells". Spinach, like other vegetables, contains many substances, not only different carotenoids but also vitamin C, folates and flavonoids that may be involved and/or be responsible for the action observed.

With the study design adopted, where the intake of fruit and vegetables was strictly controlled for 7 weeks, it would seem that even just the addition of one vegetable rich in antioxidants could exert a protective effect possibly modulating "one factor" of the equilibrium of the very complex antioxidant system. However the mechanisms involved are still not clear.

Recently Pool-Zobel et al. [29] hypothesized that the reduced DNA damage in lymphocytes following tomato and carrot juices intervention, but not spinach, could be due to the increase in cytosolic GSTP1 (an isoform of glutathione S-transferase) and DNA repair proteins, suggesting a role of vegetable juices in modulating gene expression.

We cannot come to any real conclusions about the interactions between different carotenoids as our study was not concerned with this, and also because basal values may have affected the results; however, consuming spinach and tomato together seems to improve lutein absorption in cells. In fact the increase of lutein was significant only after spinach + tomato puree consumption, even though tomato is not a good source of this carotenoid. There are few data in the literature about the availability of lutein and other carotenoids in cells. Consequently, to improve knowledge about the role of antioxidants from foods, further studies are necessary on their concentration in specific cells and/or tissues.

In conclusion, we have demonstrated that the consumption of carotenoid-rich vegetables gives protection against oxidative stress, even for a short period of time. As it is difficult to identify the specific contribution of each single compound, it would be more useful to underline the importance of consuming a diet rich in vegetables, and not to support supplements as a way of improving protection from oxidative stress.

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