

RESEARCH ARTICLE

Effect of 10-day broccoli consumption on inflammatory status of young healthy smokers

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Abstract

This study evaluated the effects of 10-day broccoli (250 g/day) intake on dietary markers and markers of inflammations in young male smokers. A dietary intervention study with a repeated measures crossover design was conducted. Circulating levels of carotenoids, folate, C-reactive protein (CRP), tumor necrosis factor alpha (TNF- α), interleukin 6 (IL-6), interleukin 6 receptor (IL-6sR) and adiponectin were measured. Broccoli intake significantly increased plasma levels of folate (+17%) and lutein (+39%), while no significant effect was observed for TNF- α , IL-6, IL-6sR or adiponectin. Plasma CRP decreased by 48% (post-hoc analysis, $p < 0.05$) following broccoli diet; this resulted to be independent from the plasma variations in lutein and folate. An inverse correlation between lycopene, TNF- α and IL-6sR was observed at baseline. In conclusion, broccoli consumption may reduce CRP levels in smokers, consistent with epidemiologic observations that fruit and vegetable intake is associated with lower circulating CRP concentrations.

Keywords

Brassica vegetables, C-reactive protein, dietary intervention, humans, inflammation

History

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Introduction

Cruciferous vegetables are rich sources of glucosinolates (precursors of isothiocyanates, ITCs), flavonoids (e.g. kaempferol, quercetin, isorhamnetin), carotenoids, vitamins (e.g. vitamin C, K1, folate), minerals (e.g. selenium, calcium, magnesium), lipoic acid, protease inhibitors and fiber (Moreno et al., 2006). These compounds may be largely responsible for the cardioprotective and chemopreventive activity of cruciferous vegetables, as reported by numerous epidemiologic studies (Van Poppel et al., 1999; Verhoeven et al., 1996). The protective actions of cruciferous components are mediated by different mechanisms, such as modulation of detoxifying enzymes and cell signalling (Conaway et al., 2002; Zhang, 2004); control of cell cycle and induction of apoptosis (Conaway et al., 2002); protection against oxidative damage (Verhagen et al., 1997); and anti-inflammatory activities (Heiss et al., 2001; Zhang, 2004). The latter is likely of utmost importance, because chronic inflammation correlates with endothelial dysfunction, insulin resistance and oxidative stress, therefore increasing the risk of cancer, diabetes, cardiovascular disease and other pathologies such as arthritis, pulmonary diseases, neurological diseases (such as Alzheimer's disease) and autoimmune diseases (Aggarwal et al., 2006; Danesh et al., 2000; Giugliano et al., 2006; Pepys & Hirschfield, 2003).

Many environmental factors, e.g. viruses, bacteria, pollutants, cigarette smoke and other stressors contribute to increase inflammation in the body through the action of both reactive oxygen species and specific molecules such as interleukins and chemokines (Aggarwal et al., 2006).

Diet can modulate inflammation chiefly by limiting the production of pro-inflammatory eicosanoids and by restoring the proper balance of pro- and anti-inflammatory cytokines (Giugliano et al., 2006). Indeed, adequate intakes of fruits, vegetables, nuts, whole grains and long-chain omega-3 fatty acids are associated with lower levels of inflammation, while dietary patterns high in refined starches, sugars, saturated and trans-fatty acids and poor in antioxidants, fiber, and omega-3 fatty acids lead to the opposite situation (Ajani et al., 2004; Clifton, 2003; Gao et al., 2004; Mozaffarian et al., 2004; Pischon et al., 2003; Van Herpen-Broekmans et al., 2004).

While several epidemiologic and some intervention studies in humans have reported positive effects of fruit and vegetable consumption on the inflammatory status, no specific information is available on the association between Brassicaceae intake and inflammation in humans (Ajani et al., 2004; Esmailzadeh et al., 2006; Gao et al., 2004; Jacob et al., 2008; Sanchez-Moreno et al., 2003; Van Herpen-Broekmans et al., 2004; Watzl et al., 2005). We have previously demonstrated that the intake of broccoli (250 g/d for 10 days) is able to affect markers of oxidative stress (Riso et al., 2010). In the present paper, we report on five validated biomarkers of inflammation, i.e. two visceral proteins [C-reactive protein (CRP) and adiponectin], two major cytokines [tumor necrosis factor alpha (TNF- α) and interleukin 6 (IL-6)], and one cytokine receptor (IL-6sR) that we analyzed in samples from the same intervention study.

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Materials and methods

Broccoli preparation and characterization

“Marathon” broccoli (*Brassica oleracea* L. var *italica*) from a single production batch was kindly provided by Di Stasi Company (Basilicata, Italy). Broccoli was harvested in a single session, shipped to Milan, cleaned, chopped, blanched to inactivate enzymatic oxidation, chilled, and frozen at -20°C until consumption, when they were steamed for 15 min.

Steamed broccoli was analyzed for their carotenoid, vitamin C and ITC content (Riso et al., 2009, 2010). Each portion (250 g) of broccoli provided 3.1 mg of lutein, 1.4 mg of β -carotene, 146 mg of vitamin C and 108 μmol of total ITCs.

Experimental design

The study was approved by the Local Ethic Committee and is in accordance with the Declaration of Helsinki. All participants gave informed consent to the study.

Subjects were deprived of Brassica vegetables 10 days before experimentation. The full experimental design has been previously described (Riso et al., 2010). Briefly, healthy male volunteers were recruited from within the students of the University of Milan. Subjects were smokers (>10 cigarettes/day) and had homogeneous eating habits, as verified by an interview. Subjects with recent histories of injuries, infection, acute inflammation, allergy or usage of anti-inflammatory drugs were excluded from this study.

The dietary intervention was performed using a repeated measure crossover design. Participants were randomly divided into two groups: group 1 was assigned to the sequence broccoli diet/wash-out/control diet, whereas group 2 followed the sequence control diet/wash-out/broccoli diet. Each analysis was separated by 15 days of wash-out period.

Every day broccoli was steam-cooked and portioned (250 g) into appropriate food containers that were given to the subjects to keep under refrigeration till consumption. On Fridays, subjects were given two extra portions of broccoli to eat during the weekend. During the trial, subjects were instructed to maintain their normal dietary and lifestyle habits (as declared before enrollment) but to abstain from consuming Brassica vegetables and other carotenoid and folate-rich food sources. For this reason, during the experimental period, each subject received a complete list of foods high in these bioactives to be avoided. A 3-day food record was scheduled randomly during the two experimental periods and a 24-h record of food consumption was kept by each volunteer one day before blood collection to check compliance to the dietary instructions. In particular, subjects did not consume other brassica vegetables or other products in the list of foods to be avoided.

At the beginning and at the end of each treatment period, fasting venous blood samples were collected early in the morning after an overnight fast. Blood samples were collected and immediately processed to obtain plasma and serum. Analyses of inflammatory markers were performed in samples deriving from 17 out of 27 subjects [age 21.8 ± 2.7 y and body mass index (BMI) $21.8 \pm 1.9 \text{ kg/m}^2$], who were involved in a previous study (Riso et al., 2010) and gave their consent to increase the amount of blood collected in order to perform further analysis.

Assessment of inflammatory markers

Plasma TNF- α , IL-6, IL-6sR and adiponectin concentrations were determined by means of commercially available immunoassay kits (“Quantikine HS Human TNF- α Immunoassay”, “Quantikine HS Human IL-6 Immunoassay”, “Quantikine Human IL-6sR Immunoassay” and “Quantikine Human

Adiponectin Immunoassay”, respectively) following the instructions provided by the manufacturer (R&D Systems, Inc., Minneapolis, MN). These test kits employ a solid-phase enzyme-linked immunosorbent assay on a microplate coated with a monoclonal antibody specific for each marker.

Serum CRP was measured with the “PCR Latex Autom”, a commercially available kit based on quantitative immunoturbidimetric determination against control standards, according to the manufacturer’s instructions (Sentinel Diagnostics, Milano, Italy).

Determination of dietary markers in plasma

Carotenoids and folate were analyzed in plasma as previously reported (Riso et al., 2009). These dietary markers were selected because their blood concentrations increased after 10 days of broccoli consumption. As regard ITCs, we have previously reported an increase of their plasma concentrations following 10-day broccoli intake, providing an amount of about 200 μmol ITCs (Riso et al., 2009). In the present study, we did not perform the analysis again due to the limited amount of available blood sample.

Statistical analyses

Statistical analysis was carried out with the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL) release 17.0 for Windows.

The normal distribution of the variables was verified by means of the Shapiro–Wilk test. Skewed data were normalized by logarithmic transformation. Analysis of variance (ANOVA) was used to evaluate the effect of daily broccoli consumption on the variables under study. A repeated measure ANOVA with the sequence of diets (broccoli then control diet or control then broccoli diet) as the independent factor was performed to evaluate whether a significant carry-over effect was present. When no carry-over effect was observed, data were matched and analyzed with ANOVA considering diets (broccoli and control) and time (before and after treatments) as dependent factors. Differences between means were further evaluated by the Least Significant Difference test (LSD).

Relationships between markers of inflammation and dietary markers at baseline were determined using the Pearson correlation test. The level of significant was set at $p < 0.05$. Samples measured with enzyme-linked immunosorbent assay methods were run in duplicate while those obtained by HPLC in triplicate. Data are presented as mean and standard deviation (SD).

Results

Baseline characteristics of the subjects

Table 1 shows the subjects’ characteristics at recruitment, including their baseline plasma concentrations of the markers under study. All the data were within the range of normality.

Effect of broccoli consumption on inflammatory markers

Results for the markers of inflammation that we evaluated are reported in Table 2. On the whole, two-way ANOVA did not show a significant effect of dietary treatment (broccoli versus control diet) in the circulating concentrations of adiponectin, TNF- α , IL-6 and IL-6sR but an effect close to the significance for the CRP in the variable time ($p = 0.054$). In this regard, *post-hoc* comparisons (LSD test) revealed that CRP concentrations significantly decreased (-48% , $p = 0.014$) in serum only following broccoli consumption but not after control diet.

Effect of broccoli consumption on dietary markers

Results on blood concentration of carotenoids and folate are reported in Table 3. There was a significant time \times treatment interaction for folate, lutein and zeaxanthin ($p = 0.036$, $p = 0.017$ and $p = 0.003$, respectively). In particular, *post-hoc* comparison revealed a significant increase for folate (+17%, $p = 0.010$) and lutein (+39%; $p = 0.0009$), and a significant decrease (–25%; $p = 0.04$) for zeaxanthin after broccoli consumption. No significant effect of either broccoli or control diets on lycopene and alpha- and beta-carotene concentrations was observed, in agreement with what we previously reported for the whole group of subjects (Riso et al., 2010).

The variation in CRP levels following the broccoli intervention resulted to be independent from the variations in lutein

($r = -0.01$, $p = 0.63$) and folate ($r = -0.17$, $p = 0.15$) plasma concentrations (data not shown).

Correlation between dietary and inflammatory markers at baseline

The correlations among dietary markers and markers of inflammation at baseline are reported in Table 4. Overall, a positive correlation at baseline between lutein, zeaxanthin, beta-cryptoxanthin and, alpha and beta carotene was observed. Beta carotene was also positively associated with beta-cryptoxanthin and lycopene.

Moreover, the analysis pointed out an inverse association between the concentration of lycopene and TNF- α ($r = -0.607$; $p = 0.010$) and IL-6sR ($r = -0.592$; $p = 0.012$) at baseline. Furthermore, an inverse correlation has been also observed between CRP and IL-6sR ($r = -0.624$, $p = 0.007$).

Discussion

There is increasing interest in the role of diet and specific food components in the prevention and/or mitigation of systemic inflammation (Galland, 2010). We assessed the effect of broccoli intake on inflammatory markers. This is especially interesting in subjects who are more prone to develop chronic, low-grade inflammation, such as smokers.

Indeed, numerous studies evaluated the impact of smoking on inflammation; even though the issue is still controversial a dose-dependent correlation between CRP levels and smoking habits has been suggested (Tonstad & Cowan, 2009). In the present study, we observed following 10-day of broccoli diet a tendency toward a decrease of serum CRP, which is consistent with the epidemiologic observations that vegetable intake is associated with low circulating CRP levels (Esmailzadeh et al., 2006; Helmersson et al., 2009; Holt et al., 2009; Wannamethee et al., 2006).

While large increases of CRP in clinical settings are considered as indicators of acute inflammation and infection, it is also important to take into account small upward variations in CRP, which could reflect low-grade chronic inflammation

Table 1. Subjects' characteristics at recruitment ($n = 17$)^a.

Clinical variables	Value
Age (years)	21.8 \pm 2.7
BMI (kg/m ²)	21.8 \pm 1.9
Smoke (cigarettes/day)	13 \pm 2
Adiponectin (μ g/mL)	7.5 \pm 3.6
CRP (mg/dL)	0.2 \pm 0.2
TNF- α (pg/mL)	0.8 \pm 0.2
IL-6 (pg/mL)	1.5 \pm 1.0
IL-6 sR (pg/mL)	379 \pm 91
Folate (nmol/L)	9.7 \pm 3.6
Lutein (μ mol/L)	0.4 \pm 0.1
α -carotene (μ mol/L)	0.1 \pm 0.1
β -carotene (μ mol/L)	0.3 \pm 0.3
Lycopene (μ mol/L)	0.2 \pm 0.1
Zeaxanthin (μ mol/L)	0.04 \pm 0.03
β -cryptoxanthin (μ mol/L)	0.2 \pm 0.1

BMI = Body mass index, CRP = C-reactive protein, TNF- α = Tumor necrosis factor-alpha, IL-6 = Interleukin-6, IL-6sR = Interleukin-6 soluble receptor.

^aData are reported as means \pm SD (Standard Deviation).

Table 2. Circulating levels of inflammatory markers as determined before and after each treatment ($N = 17$)^a.

	Before broccoli diet	After broccoli diet	Before control diet	After control diet	Time effect p	Interaction p	Treatment effect p
CRP (mg/dL)	0.18 \pm 0.20 ^a	0.10 \pm 0.10 ^b	0.12 \pm 0.10 ^{ab}	0.12 \pm 0.09 ^{ab}	0.054	0.227	0.899
TNF- α (pg/mL)	0.77 \pm 0.20	0.79 \pm 0.19	0.78 \pm 0.17	0.84 \pm 0.23	0.250	0.616	0.635
IL-6 (pg/mL)	1.57 \pm 1.15	1.74 \pm 1.69	1.71 \pm 1.37	1.55 \pm 1.13	0.911	0.674	0.894
IL-6 sR (pg/mL)	369 \pm 102	381 \pm 105	375 \pm 89	377 \pm 100	0.368	0.491	0.983
Adiponectin (ng/mL)	73.1 \pm 39.5	73.7 \pm 41.0	75.3 \pm 36.3	71.0 \pm 31.0	0.487	0.438	0.841

CRP = C-reactive protein, TNF- α = Tumor Necrosis Factor α , IL-6 = Interleukin 6, IL-6sR = Interleukin 6 soluble Receptor.

^aData are reported as means \pm SD. Data with different letters in the same row are significantly different ($p < 0.05$; LSD test).

Table 3. Circulating levels of dietary markers as measured before and after each treatment ($N = 17$)^a.

	Before broccoli diet	After broccoli diet	Before control diet	After control diet	Time effect p	Interaction p	Treatment effect p
Folate (nmol/L)	10.1 \pm 3.9 ^a	12.6 \pm 3.5 ^b	11.7 \pm 7.0 ^{ab}	11.4 \pm 4.1 ^{ab}	0.002	0.036	0.811
Lutein (μ mol/L)	0.45 \pm 0.19 ^a	0.59 \pm 0.21 ^b	0.43 \pm 0.22 ^a	0.45 \pm 0.22 ^a	<0.001	0.017	0.180
β -carotene (μ mol/L)	0.32 \pm 0.30	0.32 \pm 0.27	0.29 \pm 0.29	0.31 \pm 0.36	0.486	0.628	0.641
α -carotene (μ mol/L)	0.10 \pm 0.10	0.09 \pm 0.09	0.11 \pm 0.10	0.13 \pm 0.17	0.399	0.398	0.672
Zeaxanthin (μ mol/L)	0.08 \pm 0.04 ^a	0.06 \pm 0.03 ^b	0.06 \pm 0.04 ^{ab}	0.07 \pm 0.05 ^{ab}	0.102	0.003	0.766
β -cryptoxanthin (μ mol/L)	0.36 \pm 0.26	0.36 \pm 0.40	0.43 \pm 0.48	0.37 \pm 0.42	0.105	0.788	0.990
Lycopene (μ mol/L)	0.29 \pm 0.11	0.26 \pm 0.09	0.28 \pm 0.12	0.29 \pm 0.12	0.922	0.356	0.934

Data with different letters in the same row are significantly different ($p < 0.05$; LSD test).

^aData are reported as means \pm SD.

Table 4. Correlations between inflammatory and dietary markers at baseline.

	log α -carotene	log β -carotene	log β -cryptoxanthin	log CRP	log folate	log IL-6	IL-6sR	Lycopene	Lutein	log TNF- α
log β -carotene	0.717**									
β -cryptoxanthin	0.543*	0.530*								
log CRP	−0.043	0.385	0.345							
log folate	0.369	0.087	0.303	−0.225						
log IL-6	0.128	0.030	0.046	0.157	−0.135					
IL-6sR	−0.171	−0.432	0.022	−0.624**	0.195	−0.042				
Lycopene	0.384	0.501*	0.072	0.253	0.155	−0.176	−0.592*			
Lutein	0.694**	0.654**	0.691**	0.133	0.369	0.323	−0.077	0.272		
log TNF- α	−0.102	−0.105	0.161	−0.035	−0.046	−0.030	0.221	−0.607**	−0.222	
log zeaxanthin	0.477	0.603*	0.517*	0.260	0.192	0.390	0.070	0.219	0.791**	−0.177

CRP = C-reactive protein, IL-6 = interleukin 6, IL-6sR, = Interleukin 6 soluble receptor, TNF- α = Tumor necrosis factor alpha.

*Significant for $p \leq 0.05$.

**Significant for $p \leq 0.01$.

(Tonstad & Cowan, 2009). Therefore, from a preventive viewpoint, it becomes important to investigate the modulatory effect of dietary treatment on this parameter. Interestingly, we observed a small, but significant, CRP reduction following broccoli intake after LSD test even though the initial CRP levels of smokers were within the normal range. It is noteworthy that studies on early exposure to tobacco smoke (e.g. in adolescents) have reported higher levels of CRP with respect to nonsmokers, suggesting that its modulation is not necessarily dependent on long-term exposure to the noxious stimulus (O'Loughlin et al., 2008).

In terms of dietary modulation, recent epidemiological studies found inverse associations between plasma CRP concentrations and the intake of both fruits and vegetables (Esmailzadeh et al., 2006); the intake of fruit, vitamin C and folate (Holt et al., 2009; Wannamethee et al., 2006); and dietary intakes of vitamin C and vitamin E (Helmertsson et al., 2009). However, while a high overall intake of vegetables and fruits appears to be associated with lower levels of inflammation, the evidence for specific effects of single vegetable and fruit varieties is currently not conclusive (Calder et al., 2011; Galland, 2010). This includes the lack of information on the *in vivo* anti-inflammatory effect of cruciferous vegetables. However, it is noteworthy that, through the use of a specific statistical method, a nested case control study extrapolated a dietary pattern that was strongly associated with markers of inflammation and endothelial dysfunction (high in sugar-sweetened soft drinks, refined grains, diet soft drinks and processed meat) (Schulze et al., 2005). Remarkably, it was found that a high intake of cruciferous vegetables and yellow vegetables (but also wine and coffee) reduced inflammatory markers. Moreover, this pattern strongly predicted risk of type-2 diabetes, independent of body mass index and other diabetes risk factors (Schulze et al., 2005).

Data from human dietary intervention studies are very limited and conflicting (Calder et al., 2011). For example, significant CRP reductions were observed in subjects consuming diets high in vegetables and fruits (Watzl et al., 2005), red orange juice (Buscemi et al., 2012), orange and blackcurrant juice (Dalgård et al., 2009), mangosteen juice (Udani et al., 2009), or almonds (Rajaram et al., 2010). Conversely, no variations were observed after interventions with a strawberry drink (Basu et al., 2009), carrot juice (Potter et al., 2011), lutein and zeaxanthin-rich food (Graydon et al., 2012), dried cranberries (Vidlar et al., 2010), or cranberry juice (Basu et al., 2011).

We did not find inverse correlations between CRP levels and markers of broccoli consumption, namely lutein, β -carotene and folate. However, it is worth mentioning that broccoli is also a good source of polyphenols and vitamin C, in addition to

glucosinolates. These molecules have been suggested as important anti-inflammatory agents. For example, in a US population survey, flavonoid intake was inversely associated to CRP concentration, even after adjustment for total fruit and vegetable consumption (Chun et al., 2008).

Broccoli intervention did not produce significant modulations of the other inflammatory markers considered, namely IL-6, IL-6sR, TNF- α and adiponectin.

Concerning dietary markers, a limitation of the study is the absence of plasma concentration measurements of ITCs. However, we previously reported that a 10-day broccoli diet, providing the same amount of ITCs, was able to increase their plasma concentrations in a group of healthy volunteers (Riso et al., 2009). In the present study, we documented that broccoli intervention increased plasma concentration of folate and lutein. The extent of increase of these dietary markers could be dependent on initial levels that were low in our smoker volunteers.

A significant positive correlation was observed between lutein, zeaxanthin, beta-cryptoxanthin, alpha and beta carotene. These carotenoids are present combined in fruit and vegetable; thus their concentrations are indicative of fruit and vegetable consumption. The correlation between markers of inflammation and dietary markers at baseline was also investigated. No correlation was found between CRP and dietary markers; on the contrary basal lycopene concentrations were inversely correlated with TNF- α levels ($p < 0.05$). These data are in agreement with previous studies, in which the intake of a tomato-based drink (which increased plasma lycopene concentrations) determined a significant decrease of TNF- α levels as compared to placebo-controls (Riso et al., 2006). In literature, a lower adherence to a Mediterranean dietary pattern (resulting in low circulating plasma levels of carotenoids, comprising lycopene, vitamin A and vitamin E) was associated to higher TNF- α levels in a group of Italian subjects (Azzini et al., 2011). These two pieces of evidence suggest the possible implication of lycopene (*per se* or as marker of specific food items) in the modulation of TNF- α .

Before the current findings can be extrapolated to the general population, it needs to be underscored that we studied young smokers; therefore, they might have not been exposed to oxidative stress through prolonged periods of time, as suggested by the observation that their circulating concentrations of inflammatory markers were within the normal range at the beginning of the study. Supporting this hypothesis is the observation that the decrease in CRP levels induced by broccoli was clear in those subjects who had slightly higher baseline levels as compared to the other volunteers.

Conclusions

A relatively short period of daily broccoli consumption seems to be able to affect CRP circulating levels in young healthy smokers, consistent with the epidemiological observation of a positive modulatory effect of fruit and vegetables. Such data can contribute to improve our understanding of the potential benefits associated with consumption of whole foods on selected inflammatory markers. However, considering the small group of subjects enrolled, further studies are necessary to verify the effect of broccoli or their bioactive components in the modulation of CRP and other markers of inflammation which were not affected in the present study.

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Declaration of interest

Nothing to disclose.

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