Effect of Broccoli Intake on Markers Related to Oxidative Stress and Cancer Risk in Healthy Smokers and Nonsmokers

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Cruciferous vegetables (CVs) have been widely studied for their anticarcinogenic properties. The aim of the present study was to evaluate the protective effect of broccoli intake in smokers and nonsmokers.

Twenty young healthy males (10 smokers and 10 nonsmokers) were randomized in a cross-over design and received a portion of broccoli (200 g) or maintained a controlled diet for 10 days each. The two periods were separated by a wash out period (20 days). Blood samples were collected at 0, 10, 30, and 40 days and used for the evaluation of DNA damage, insulin-like growth factor-I (IGF-I) and histone deacetylase (HDAC). Ex vivo protection from H2O2-induced DNA damage and endogenous DNA damage were evaluated in lymphocytes by means of the comet assay. Strand breaks decreased significantly after the broccoli diet in smokers as well as in nonsmokers (−22.2%; P < 0.0001), whereas oxidized purines decreased significantly only in smokers (−51.0%; P < 0.0001). Broccoli intake did not modify HDAC activity and IGF-I serum levels. Our results strengthen the importance of consuming CVs to increase cell protection against DNA damage. Future investigation, with different amount of broccoli and/or different time of exposure, is needed to understand the lack of effect on HDAC activity and IGF-I levels.

INTRODUCTION

Various data support the consumption of cruciferous vegetables (CVs) such as broccoli, cabbage, cauliflower, watercress, and Brussels sprouts as a protective factor against cancer development. In this regard, an overview of the epidemiological data was given by van Poppel et al. (1) who summarized the results of 6 cohort studies and 74 case-control studies, supporting an inverse association between the consumption of CVs and risk of several types of cancers.

CVs contain a range of potentially anticarcinogenic dietary factors including carotenoids, vitamin C, fiber, and flavonoids (2). In addition, they contain substantial amounts of glucosinolates, sulfur-containing glycosides that are hydrolyzed to isothiocyanates (ITCs), and indoles when the raw vegetables are chewed or otherwise macerated (3).

The role of ITCs on the protection against cancer development has been investigated in both cell culture and animal models (4,5).

The chemopreventive properties of such compounds can be attributed to different mechanisms including alteration in the activity of detoxifying enzymes by induction of phase 2 enzymes and inhibition of phase 1 enzymes (6), radical and electrophil scavenger activity, induction of apoptosis, and regulation of cell cycle (7–9).

Moreover some studies have suggested a significant effect of sulforaphane, an ITC derived from glucoraphanin present in broccoli, on the inhibition of histone deacetylase (HDAC) activity in cultured cancer cells and in animal models (10,11).

HDAC plays a key role in carcinogenesis, and it has been considered as one of the target molecules for cancer therapy (12). Compounds that inhibit HDACs have the potential to induce the transcription of tumor suppressor proteins that promote differentiation and apoptosis in precancerous cells. Very recently it was found that the intake of broccoli sprouts was able to inhibit...
HDAC activity in peripheral mononuclear cells of healthy human volunteers (13). Confirmation of such a result would be of great importance for the understanding of the role and mechanism of action of compounds present in cruciferous vegetables.

Under discussion is also the potential role of the intake of this class of vegetables in the protection against DNA damage. Although the effects of CVs consumption on DNA damage have been studied in several in vitro (14,15) and in vivo studies on rats, only few studies on humans have been carried out (16–19). In this context, the results from a recent intervention study demonstrated a significant protective effect of watercress consumption consumed for 8 wk on oxidatively induced DNA damage (20).

The aim of the present study was to investigate whether the regular intake of broccoli could modulate biomarkers of DNA damage (endogenous and oxidatively induced) and affect HDAC activity in healthy smokers and nonsmokers. Moreover, in view of the possible implication of insulin-like growth factor-I (IGF-I) in the etiology of chronic diseases, including cancer, and the modulatory effect of several dietary factors on it, we evaluated IGF-I serum levels throughout the whole intervention.

MATERIAL AND METHODS

Subjects and Study Design

Twenty healthy males were selected from the students of the University of Milan and enrolled on the basis of their food habits, evaluated by means of a questionnaire in order to have an homogeneous group of volunteers for fruit and vegetable intake (subjects characteristics are reported in Table 1). Subjects with high (>5 portions/day) or low (<2 portions/day) intake of fruit and vegetables and those who followed macrobiotic diets on weekends, excluded. Smokers were included in the study if they smoked more than 10 cigarettes/day. The study was approved by the Local Ethical Committee, and informed consent was signed by each participant.

A cross-over design was scheduled. Smokers and nonsmokers were randomly divided into two groups of ten subjects each (each group included 5 smokers and 5 nonsmokers): Group 1 was assigned to the sequence broccoli diet, wash out, controlled diet, whereas Group 2 followed the sequence controlled diet, wash out, broccoli diet. Broccoli and controlled diet were each 10 days long, and they were separated with a 20-day wash out period (free diet). During the controlled diet, subjects could follow their habitual diet avoiding the intake of cruciferous vegetables. A list of unallowed vegetables was given to each subject.

During the period of supplementation with broccoli, subjects received a daily portion (200 g) of “Marathon” broccoli (Brassica oleracea L. var italica). The vegetables arrived at the laboratory one day after harvesting and were immediately processed at the Experimental Institute for Agricultural Product Technologies (IVTPA, Milan). Broccoli florets were handled gently, excised from the main stem, immediately blanched, and after cooling at room temperature for some minutes, frozen at −25°C.

During the trial, every day a defined amount of broccoli was steamed for 15 min and portioned into appropriate food containers. Each portion of broccoli provided carotenoids (about 5 mg lutein and β-carotene) and an amount of vitamin C ranging from about 150 mg (when consumed within 2 h from steaming) to about 60 mg (when consumed after 48 h cold storage) as previously reported (21). In addition, a single portion provided about 200 µmol PEITC equivalents, where PEITC is the acronym of phenethyl isothiocyanate, a naturally occurring compound found in some cruciferous vegetables, which is used as reference standard to quantify total ITCs by the cyclocondensation reaction (22).

Generally, subjects consumed the portion of broccoli for lunch time at the laboratory together with their meal. On Friday, subjects were given two portions to eat over the weekend. In this case, the intake of vitamin C could be lower than during the week, as commented before. However, the intervention was scheduled so that subjects had only one weekend in the 10-day period and not close to the day of blood collection.

### TABLE 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All</th>
<th>Smokers</th>
<th>Nonsmokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. males</td>
<td>20</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>23.0 ± 1.8</td>
<td>22.9 ± 1.5</td>
<td>23.1 ± 2.2</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>24.1 ± 2.9</td>
<td>25.0 ± 2.8</td>
<td>23.2 ± 2.7</td>
</tr>
<tr>
<td>Background strand breaks (% DNA in tail, PBS)</td>
<td>11.3 ± 2.6</td>
<td>10.7 ± 2.1</td>
<td>11.8 ± 3.0</td>
</tr>
<tr>
<td>H₂O₂-induced strand breaks (% DNA in tail)</td>
<td>63.0 ± 13.0</td>
<td>62.8 ± 14.0</td>
<td>63.3 ± 12.7</td>
</tr>
<tr>
<td>Background strand breaks (% DNA in tail, EB)</td>
<td>21.3 ± 7.4</td>
<td>23.0 ± 7.1</td>
<td>19.5 ± 7.7</td>
</tr>
<tr>
<td>FPG-sensitive sites (% DNA in tail)</td>
<td>15.2 ± 6.7</td>
<td>13.0 ± 4.4</td>
<td>18.0 ± 8.4</td>
</tr>
<tr>
<td>IGF-I (ng/ml)</td>
<td>293.9 ± 57.0</td>
<td>262.7 ± 44.9</td>
<td>325.5 ± 51.1</td>
</tr>
<tr>
<td>HDAC activity (pmol/ul/10⁶ cells)</td>
<td>34.9 ± 30.3</td>
<td>37.2 ± 29.4</td>
<td>32.6 ± 32.6</td>
</tr>
</tbody>
</table>

*Abbreviations are as follows: BMI, body mass index; PBS, phosphate-buffered solution; FPG, formamidopyrimidine DNA glycosylase; IGF-I, insulin-like growth factor-I; HDAC, histone deacetylase.*
Blood samples were collected at the beginning and at the end of each period of supplementation (0, 10, 30, 40 days) early in the morning after overnight fasting. Samples were drawn into evacuated tubes with heparin as an anticoagulant (to obtain lymphocytes) or without an anticoagulant (to obtain serum).

Lymphocytes were separated by density gradient centrifugation of whole blood with Histopaque 1077 (Sigma Chemicals Co., St. Louis, MO).

The lymphocyte layer was removed from the gradient, washed with PBS, and used immediately for the determination of whole blood with Histopaque 1077 (Sigma Chemicals) or without an anticoagulant (to obtain serum).

Estimation of Strand Breaks Using the Comet Assay

The resistance of lymphocytes to oxidative stress was evaluated using the comet assay as previously reported (23). Separated lymphocytes were fixed with agarose on fully frosted slides (Richardson Supply Co., London, UK). Two slides for each subject were prepared. One sample was subjected to a H₂O₂ treatment (using a solution of H₂O₂ 500 µmol/l in PBS) for 5 min, whereas the other one acted as a control.

Slides were put in lysis buffer for 1 h at 4°C and then were left for 40 min in electrophoresis buffer in a horizontal electrophoresis tank prior to electrophoresis in the same solution at 25 V, 300 mA for 20 min. Slides were successively neutralized, stained with ethidium bromide (2 µg/ml), washed in PBS, drained, and then covered with cover slips.

One hundred cells for each slide were electronically captured using an epifluorescence microscope attached to a high-sensitivity CCD video camera and to a computer provided with an image analysis system (Cometa 1.5; Immagini e Computer, Bareggio, Milan, Italy). Levels of strand breaks were calculated as percentage DNA in the tail. For each subject, the percentage DNA in the tail of control cells (not treated with H₂O₂) was subtracted from the percentage DNA in the tail of H₂O₂-treated cells.

Estimation of Formamidopyrimidine DNA Glycosylase Sensitive Sites

Detection of oxidized bases was performed by means of the enzyme formamidopyrimidine DNA glycosylase (FPG), which introduces breaks at sites of oxidized purines such as 8-oxo-2'-deoxyguanosine (8-oxodG). The method used for this evaluation has been previously described (24). After the lysis stage, cells were incubated at 37°C for 45 min with FPG (100 ng/ml) diluted in EB buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM Na₂EDTA, 0.2 mg/ml bovine serum albumin, pH = 8) or with EB buffer alone (control). Alkaline treatment and electrophoresis then followed. FPG-sensitive sites were calculated by subtracting the percentage DNA in the tail of control cells (treated with EB alone) from the percentage DNA in the tail of cells treated with FPG.

HDAC Activity Evaluation

HDAC activity was determined by fluorescence (HDAC Assay Kit, Upstate, Virginia) on lymphocytes samples diluted 1:25, with excitation at 360 nm and emission at 460 nm.

Samples were first incubated with the HDAC Assay substrate, allowing deacetylation of the fluorometric substrate. After incubation, the activator solution was added and released a fluorophore from the deacetylated substrate or standard. The sample was then placed into a microplate reader (Victor 3 model, Wallac) (Perkin Elmer, Waltham, MA) and fluorescence was read.

IGF-I Evaluation

Total IGF-I was determined by a radio immunoassay (RIA; Bio Source Europe, Nivelle, Belgium). The assay was performed after acid-ethanol extraction of serum in order to avoid any interference from IGF binding proteins (IGF bps). The analytical sensitivity of the assay (defined as the smallest value that can be distinguished from zero with a 95% confidence limit) was 0.25 ng/ml. The within- and between-assay coefficients of variation were 4.1% and 9.6%, respectively. Cross-reactivity with IGF-II was less than 0.2%.

Statistical Analysis

Statistical analyses were performed using the STATISTICA software (Statsoft Inc., Tulsa, OK). A repeated-measures analysis of variance (ANOVA) with sequence (broccoli diet vs. controlled diet or vice versa) as dependent factor was used in order to evaluate the presence of a carryover effect. As none was detected, data from the two groups of subjects were considered independently from the sequence of treatment and analyzed together.

ANOVA with treatment (broccoli diet vs. controlled diet) and time (before and after each treatment) as dependent factors was used to investigate the effect of broccoli consumption on cell resistance to DNA oxidative damage, levels of FPG-sensitive sites, IGF-I concentration, and HDAC activity. Data were also analyzed taking into account smoking habit as the independent factor.

Differences between means were evaluated by the LSD test. Differences were considered significant at \( P < 0.05 \).

RESULTS

Lymphocyte DNA damage (as percentage of DNA in the tail) registered in smokers and nonsmokers before and after broccoli and controlled diet consumption is reported in Table 2.

Despite a trend toward a decrease of DNA damage after both broccoli diet and controlled diet, the reduction was significant only after broccoli consumption \(( P < 0.0001)\). After 10 days of
Broccoli intake, the percentage of DNA in the tail decreased by about 22.2% as calculated by considering the variation between the percentages of DNA in the tail registered before and after broccoli consumption. Dividing subjects into smokers and nonsmokers, DNA damage decreased similarly in both groups (−23.2% in smokers and −21.2% in nonsmokers).

FPG-sensitive sites decreased significantly after the broccoli diet (Table 2). ANOVA did not reveal a significant effect of smoking status probably because there was a reduction in FPG-sensitive sites in both the groups. However, this reduction was significant (LSD test) in smokers (−51.0%; \( P < 0.0001 \)) but not in nonsmokers (−25%, nonsignificant).

Supplementation with broccoli did not affect significantly HDAC activity as compared with the controlled diet (Table 2). This lack of effect was confirmed also when data from smokers and nonsmokers were analyzed separately.

Finally, IGF-I levels remained nearly constant both in smokers and nonsmokers during the whole experimental period as clearly shown in Table 2.

**DISCUSSION**

The results of the present study indicate that broccoli consumption can increase cell resistance to oxidative stress. In fact, we obtained a decrease of \( \text{H}_2\text{O}_2 \)-induced DNA damage after 10 days consumption of 200 g broccoli/day. Comparable data have been obtained in previous studies carried out in our laboratory with other fruits and vegetables (i.e., tomatoes, spinach, oranges, etc.), providing demonstration of the protective efficacy of vegetable intake (25–27).

Moreover, return of DNA damage to initial levels after the wash-out period proves that a regular consumption of vegetables is important to guarantee a protective effect.
To our knowledge, there are no other studies in the literature investigating the medium term effects of broccoli consumption on cell DNA resistance to oxidative stress. A recent study by Gill et al. (20) found a nonsignificant decrease in H2O2-induced DNA damage (by 9.4%; \( P = 0.07 \)) after watercress supplementation (85 g for 8 wk) but significant decreases in the background level of DNA strand breaks (−17%; \( P = 0.03 \)) and in strand breaks plus oxidized purines (−24%; \( P = 0.002 \)). We also found a significant effect of broccoli consumption on FPG-sensitive sites.

The lower endogenous DNA damage found after broccoli consumption may be explained by both a reduction of the overall oxidative stress and/or a modulatory activity on the cell defense system exerted by compounds introduced with this vegetable. In fact, ITCs have been suggested to be able to modulate detoxifying enzymes such as GSTs and it cannot be excluded they may also modulate other enzymes. For example, the reduction of FPG-sensitive sites could be explained by an increase of DNA repair ability as previously suggested by Collins et al. (28) in an intervention study with kiwi fruit. However, further studies are necessary in order to better elucidate the potential role of broccoli on the repair system.

Regarding the effect of broccoli consumption on HDAC activity, we did not observe an inhibitory action. Myzak et al. (13) recently reported HDAC inhibition 3 and 6 h following a bolus ingestion of BroccoSprouts in healthy subjects. The differences with our study include the test material, that is, mature broccoli vs. sprouts, and the time frame of the experiment, that is, regular consumption vs. acute ingestion. Moreover, Myzak et al. (13) administered to 3 healthy subjects 105 mg sulforaphane, equivalent to approximately 570 g of mature broccoli, which is much more than the amount we could provide with the whole food.

As far as we know, our study is the first evaluating the effect of a dietary amount of broccoli for a relatively long period on HDAC activity in a controlled intervention study.

As research increasingly suggests the chemopreventive potential of compounds found in broccoli (29,30), future investigations with different amounts/types of cruciferous and patterns of consumption are warranted.

Some data exist in the literature suggesting a possible role of vegetable intake in the modulation of IGF-I (31,32). However, in the present study we did not find such an effect of broccoli in contrast to the decrease in IGF-I seen in our previous intervention study with a tomato product (33). This might be caused by the short period of intervention or the inefficacy of this type of food compounds on IGF-I metabolism.

In conclusion, the present study demonstrated that broccoli intake can reduce significantly markers related to oxidative stress both in smokers and nonsmokers. Despite the fact that we did not find differences between the two groups of subjects on protection from exogenous DNA damage, we found significant decreases in the levels of oxidized purines after broccoli in smokers; consequently, such subjects may particularly benefit from broccoli intake. In this context, we should underline that the volunteers involved in our study were all young (<mid-20s), and so they may have not been exposed to toxic compounds present in cigarette smoke for a very long time. This may explain some comparable results found between the two groups.

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