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Water extracts of cabbage and kale inhibit *ex vivo* H₂O₂-induced DNA damage but not rat hepatocarcinogenesis

M.A. Horst¹, T.P. Ong², A.A. Jordão Jr.³, H. Vannucchi³, F.S. Moreno²
and F.M. Lajolo¹

¹Laboratório de Química e Bioquímica de Alimentos, ²Laboratório de Dieta Nutrição e Câncer, Departamento de Alimentos e Nutrição Experimental, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, SP, Brasil

³Divisão de Nutrologia, Departamento de Clínica Médica, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil

Abstract

The chemopreventive potential of water extracts of the *Brassica* vegetables cabbage and kale was evaluated by administering their aqueous extracts in drinking water *ad libitum* to Wistar rats submitted to Ito's hepatocarcinogenesis model (CB group and K group, respectively - 14 rats per group). Animals submitted to this same model and treated with water were used as controls (W group - 15 rats). Treatment with the vegetable extracts did not inhibit ($P > 0.05$) placental glutathione S-transferase-positive preneoplastic lesions (PNL). The number of apoptotic bodies did not differ ($P > 0.05$) among the experimental groups. *Ex vivo* hydrogen peroxide treatment of rat livers resulted in lower ($P < 0.05$) DNA strand breakage in cabbage- ($107.6 \pm 7.8 \mu\text{m}$) and kale- ($110.8 \pm 10.0 \mu\text{m}$) treated animals compared with control ($120.9 \pm 12.7 \mu\text{m}$), as evaluated by the single cell gel (comet) assay. Treatment with cabbage ($2 \pm 0.3 \mu\text{g/g}$) or kale ($4 \pm 0.2 \mu\text{g/g}$) resulted in increased ($P < 0.05$) hepatic lutein concentration compared with control ($0.5 \pm 0.07 \mu\text{g/g}$). Despite the absence of inhibitory effects of cabbage and kale aqueous extracts on PNL, these *Brassica* vegetables presented protection against DNA damage, an effect possibly related to increased hepatic lutein concentrations. However, it must be pointed out that the cause-effect relationship between lutein levels and protection is hypothetical and remains to be demonstrated.

Key words: Chemoprevention; Hepatocarcinogenesis; Diethylnitrosamine; *Brassica* vegetables; DNA damage

Introduction

Over 6 million people die of cancer worldwide each year and the incidence of the disease will increase by 50% in 2020 (1). Diet appears to be one of the most important determinants of human cancer, with about one-third of all cases being attributed to dietary factors (2,3). More than 200 epidemiological studies have shown that a high consumption of fruits and vegetables protects against cancer. These inhibitory effects have been related to the ingestion of nutrients and bioactive compounds such as vitamins C and E and carotenoids, glucosinolates and flavonoids that show anticarcinogenic actions (3).

The consumption of cruciferous vegetables, particularly those of the *Brassica* genus (cabbage, kale, broccoli, Brussels sprouts, mustard, rape, turnip, rutabaga, cauliflower,

radish) has been suggested to reduce the risk of several cancers (4). In a chemoprevention clinical trial conducted in China, consumption of hot water infusions of 3-day-old broccoli sprouts containing defined concentrations of glucosinolates resulted in reduced urinary excretion of aflatoxin-DNA adducts in a subset of individuals at high risk for hepatocellular carcinoma, suggesting that sulforaphane induced carcinogen detoxification (5). In human hepatoma cells, broccoli, Brussels sprouts and cabbage water extracts induced glutathione S-transferase activity and allyl isothiocyanate inhibited cell proliferation (6).

Despite the anticarcinogenic potential of crucifers, few animal studies have been conducted *in vivo*, especially in hepatocarcinogenesis models (7,8). Recently, administration of

Correspondence: M.A. Horst, Departamento de Alimentos e Nutrição Experimental, Faculdade de Ciências Farmacêuticas, USP, Bloco 14, Av. Prof. Lineu Prestes, 580, 05508-900 São Paulo, SP, Brasil. Fax: +55-11-3815-4410. E-mail: aderuzah@usp.br

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red cabbage and Brussels sprouts extracts to rats resulted in chemoprevention of liver and colon carcinogenesis induced by heterocyclic amine 2-amino-3-methylimidazo(4,5-f)quinoline (IQ) (7). However, earlier publications showed that breakdown products of glucosinolates such as isothiocyanates, considered to be responsible for the chemopreventive properties of cruciferous vegetables (9), can also act as mutagens or tumor promoters (10).

Thus, the objective of the present study was to determine if water extracts of cabbage and kale would present chemopreventive activities when administered to rats during the initiation and promotion phases of Ito's hepatocarcinogenesis model. The parameters evaluated included hepatic preneoplastic lesions (PNL), apoptosis and *ex vivo*-induced DNA strand breakage in liver cells. Since cabbage and kale are important sources of lutein, a carotenoid with potent antioxidant capacity and chemopreventive potential against hepatocarcinogenesis (11), we also measured its hepatic concentration.

Material and Methods

Chemicals

Diethylnitrosamine (DEN), 3,3-diaminobenzidine, formaldehyde and low melting point agarose were purchased from Sigma (USA). The commercial rat diet was purchased from Purina (Brazil). Polyclonal anti-glutathione S-transferase placental form (GST-P) rabbit antibody was purchased from Medical and Biological Laboratories Co. (Japan). Secondary biotinylated antibody and streptavidin-biotin-peroxidase complex (StrepABComplex/HRP Duet, Mouse/Rabbit) were purchased from Dako (Denmark). All other chemicals were of the highest quality available.

Water extracts of *Brassica* vegetables

Cabbage and kale were purchased from a local supermarket approximately three times a week. Extracts were prepared fresh every day from raw vegetables. After washing with water, the vegetables were cut into small pieces and added to distilled water to obtain a concentration of 10% (w/v) and cabbage and kale were blended for 3 min at approximately 22°C in a commercial blender (Walita, LiqArt model, Brazil) fitted with a filter that allowed gross particles to be removed. These procedures were performed as described by Kassie et al. (7). Because the carcinogenic process is more intense in Ito's hepatocarcinogenesis model compared to the one used in Ref. 7, we doubled the concentration of *Brassica* vegetables (from 5 to 10%, w/v) used.

Animals and experimental protocol

Male Wistar rats (21 days old, ~70 g on arrival) were obtained from the colony of the Faculty of Pharmaceutical Sciences, University of São Paulo, Brazil. They were maintained in polypropylene boxes (4 rats/box) lined with autoclaved shavings, at a constant temperature (22°C),

with a 12-h light-dark cycle and received distilled water or *Brassica* water extracts and commercial diet *ad libitum*.

At the end of a 7-day acclimatization period, with the exception of 8 Wistar rats not submitted to any experimental procedure (normal group), 45 animals were randomly divided into 3 experimental groups. Two groups were treated with *Brassica* water extracts cabbage (CB group) or kale (K group) in distilled drinking water at the concentration of 10% (w/v). A group treated with only distilled water (W group) was used as control. All treatments were carried out for 8 consecutive weeks.

Two weeks after the beginning of the treatments, rats from the CB, K and W groups were submitted to Ito's hepatocarcinogenesis model (12). Hepatocyte initiation was obtained by the administration of a single intraperitoneal dose of DEN (200 mg/kg body weight) dissolved in 0.9% NaCl. After a 3-week recovery period, the animals were subjected to 2/3 partial hepatectomy for the promotion of carcinogenesis. Six weeks after DEN administration, the animals were anesthetized and euthanized by exsanguination. Food and liquid consumption and body weights were recorded daily. The study was approved by the Ethics Committee for Animal Research of the Faculty of Pharmaceutical Sciences, University of São Paulo (Protocol #76).

Immunohistochemistry for GST-P

Representative fragments of each liver lobe were fixed in Metacarn solution (60% methanol, 30% chloroform and 10% glacial acetic acid) for approximately 24 h and embedded in paraffin. Histological sections of the liver samples were used for immunohistochemical reactions in order to detect PNL (foci) positive for GST-P according to the method described by Hsu et al. (13). After removal of paraffin, endogenous peroxidase was blocked with 3% hydrogen peroxide in phosphate-buffered physiological saline (PBS) for 5 min. The sections were then incubated overnight at 4°C with a primary anti-GST-P antibody at a 1:1000 dilution in 1% bovine serum albumin. Finally, the sections were incubated for 1 h with a secondary biotinylated antibody and the streptavidin-biotin-peroxidase complex was applied. Peroxidase binding sites were detected by incubation with 3,3-diaminobenzidine (0.5%) and hydrogen peroxide (0.1%) dissolved in PBS for approximately 2 min at room temperature. Sections were counterstained with hematoxylin and eosin (H&E).

GST-P-positive hepatocyte foci with a diameter greater than 0.15 mm (14,15) were measured with the KS-300 program (Kontron Elektronik, Germany) using a Nikon (Microphot-FXA, Japan) photomicroscope connected to a microcomputer. Data are reported as GST-P-positive PNL number (n/cm² liver section) and aggregated area (mm²/cm² liver section).

Measurement of apoptosis

The liver sections submitted to immunohistochemistry

for GST-P and counterstained with H&E were also used for the identification of apoptotic bodies (AB). AB were quantified by fluorescence microscopy (16,17) using a Nikon microscope (Japan) equipped with an epifluorescence unit. This method is based on the strong eosin fluorescence of AB in H&E-stained liver tissues submitted to blue light (450-490 nm). Identification of AB was confirmed by switching the microscope system from blue to transmitted light and using the morphological criteria established by Grasl-Kraupp et al. (18). AB were represented by acidophilic bodies with fragmentation or lack of chromatin accompanied by cytoplasmic condensation and/or fragmentation. The number of AB of each group was counted in all GST-P-positive PNL and surrounding normal tissue transections within the liver sections. Apoptotic indexes are reported as the number of hepatic AB/mm² of GST-P-positive PNL or surrounding normal tissue areas.

Hepatic DNA strand breakage (comet assay) after *ex vivo* H₂O₂-induced DNA damage in rat liver cells

Hepatic DNA strand breakage was evaluated in liver samples of groups W, CB, and K previously stored at -78°C using the comet assay (19,20). The tissues were homogenized in PBS under refrigeration and the homogenate was then added to a 0.8% low-melting point agarose, homogenized and immobilized on a microscope slide previously coated with a layer of 0.5% low-melting point agarose. The homogenate was then exposed to 150 µM H₂O₂ for 1 h and 30 min (21), washed in twice-distilled water and lysed in a solution of 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% dimethylsulfoxide, and 1% Triton X-100, pH 10, at 4°C for 1 h. After lysis, the slides were briefly washed in twice-distilled water to remove excess lysis solution and placed on a horizontal electrophoresis unit filled with fresh alkaline buffer (300 mM NaOH and 1 mM EDTA, pH >13) for 20 min at 4°C for DNA unwinding. Electrophoresis was conducted at 4°C for 20 min at 25 V and 300 mA (20) and the microscope slides were neutralized in 0.4 M Tris buffer, pH 7.5. The resulting comets were stained with silver nitrate (22), with silver being used instead of ethidium bromide. Normal rat liver tissues were used as negative controls (11). The length of the comets (head + tail) was measured

using the previously described image analysis system. One hundred nucleoids per animal were randomly analyzed (50 images per microscope slide). Coded microscope slides were scored blindly. The viability of liver cells was determined indirectly by analyzing the comet images after electrophoresis (14,20). The comet image was considered to be from a non-viable cell when it presented a cloudy appearance or a very small head and a balloon-like tail (necrotic or apoptotic cells). The viability of the cell suspension was considered to be acceptable when the frequency of such images was less than 2% (23).

Lutein quantification

Hepatic lutein concentration was determined by high-performance liquid chromatography in liver samples stored at -78°C (11,24). A Shimadzu LC 9A (Shimadzu, Japan) chromatograph with a multisolvent pump system, UV-VIS detector and reverse-phase column (CLC-ODS: 5 µm, 150 x 6 mm ID) was used. The mobile phase consisted of acetonitrile-dichloromethane-methanol (70:20:10, v/v/v) at a flow of 2 mL/min. Lutein was detected at 450 nm and quantified by comparison with the retention time and peak area of a lutein standard (25).

Statistical analysis

The Sigma Stat 2.0 (Jandel, USA) program was used for statistical analysis. One-way ANOVA and the Student *t*-test were used when the results presented normal distribution and the Kruskal-Wallis test was used in the absence of normal distribution. The level of significance was set at *P* < 0.05 in all analyses.

Results

The initial and final body weights, relative liver weight, diet and water or *Brassica* extract consumption of rats submitted to Ito's hepatocarcinogenesis model are shown in Table 1. No statistically significant differences (*P* > 0.05) were observed between the W, CB and K groups regarding these parameters and all groups showed similar body weight gain. Animals from all groups only lost weight during the carcinogenic and partial hepatectomy procedures.

Table 1. Initial and final body weights, relative liver weight, diet and water or extract consumption of rats treated with water (control), cabbage, or kale and submitted to Ito's hepatocarcinogenesis model.

Group	Initial body weight (g)	Final body weight (g)	Relative liver weight (%)	Diet consumption (g·100 g body weight ⁻¹ ·day ⁻¹)	Water or extract consumption (mL·100 g body weight ⁻¹ ·day ⁻¹)
Water (N = 15)	101 ± 6	345 ± 24	2.3 ± 0.2	8.8 ± 2.5	16.0 ± 4.2
Cabbage (N = 14)	101 ± 6	338 ± 22	2.4 ± 0.2	8.3 ± 2.4	14.8 ± 4.4
Kale (N = 14)	100 ± 6	338 ± 22	2.3 ± 0.2	8.4 ± 2.4	16.4 ± 4.9

Data are reported as means ± SD. Values within the same column were not significantly different (*P* > 0.05, one-way ANOVA).

These results indicate the absence of toxic effects and good acceptability of the crucifer extracts (10% w/v in drinking water).

Table 2 shows the values obtained by morphometric quantification of the number and aggregated area of GST-P-positive PNL of W, CB and K animals. No statistically significant differences were observed between groups regarding these parameters. This absence of differences

Table 2. Morphometric analysis of GST-P-positive PNL of rats treated with water (control), cabbage, or kale and submitted to Ito's hepatocarcinogenesis model.

Group	Number of PNL/cm ²	PNL aggregated area (mm ² /cm ²)
Water (N = 15)	16 ± 8	0.41 ± 0.23
Cabbage (N = 14)	20 ± 6	0.49 ± 0.15
Kale (N = 14)	18 ± 11	0.37 ± 0.20

Data are reported as means ± SD. GST-P = glutathione S-transferase placental form; PNL = preneoplastic lesions. Values within the same column were not significantly different ($P > 0.05$, Kruskal-Wallis test).

Table 3. Comet length of normal rat livers and rat livers of the water (control), cabbage, or kale groups submitted to *ex vivo* treatment with 150 μM H₂O₂.

Group	Number of rats	Length of comets (μm)
N	8	93.8 ± 7.1
W	15	120.9 ± 12.7 ^a
CB	14	107.6 ± 7.8 ^b
K	14	110.8 ± 10.0 ^b

Data are reported as means ± SD. N = normal animals; negative comet assay controls; W = water; CB = cabbage; K = kale. ^a $P < 0.05$ compared to N group (one-way ANOVA followed by the Student *t*-test). ^b $P < 0.05$ compared to W group (one-way ANOVA followed by the Student *t*-test).

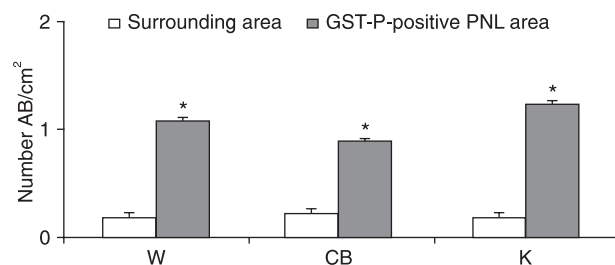


Figure 1. Quantification of apoptotic bodies (AB). W = water (control); CB = cabbage; K = kale; GST-P = glutathione S-transferase placental form; PNL = preneoplastic lesions. Data are reported as means ± SD. * $P < 0.05$ compared to the respective surrounding area (one-way ANOVA followed by the Student *t*-test).

could be eventually due to the high variability of data, with SD of about 40-60%. However, this did not seem to be the case since in a similar study (26), also using Ito's hepatocarcinogenesis model, chemopreventive activities of allyl sulfide were observed in an experiment with data variability also around 44-60% (SD).

Figure 1 shows the apoptotic index of normal areas surrounding GST-P-positive PNL and of the same areas of W, CB, and K animals. No statistically significant differences in apoptotic index were observed between the W, CB, and K groups (in normal tissue areas surrounding GST-P-positive PNL or in the GST-P-positive PNL areas themselves). However, the apoptotic index of the GST-P-positive PNL area was higher ($P < 0.05$) than that of the respective surrounding normal tissue areas in all groups. These results are consistent with the fact that apoptosis increases during hepatocarcinogenesis (27).

Table 3 presents the comet data of normal rat livers and of the entire rat livers of the W, CB and K groups submitted to *ex vivo* treatment with 150 μM H₂O₂. Liver samples from W rats challenged with 150 μM H₂O₂ showed comets of increased ($P < 0.05$) length compared to those observed in unchallenged ones (normal animals; negative comet assay controls). Liver samples from CB and K animals challenged with 150 μM H₂O₂ presented smaller comets ($P < 0.05$) than those observed in similarly challenged W group livers.

Finally, Figure 2 shows the hepatic lutein concentrations of W, CB and K rats. Animals that received *Brassica* extracts (CB and K groups) presented 4- and 8-fold increased hepatic levels of lutein, respectively, compared to animals that received only water (W group). K animals presented significantly higher hepatic levels of lutein compared to CB animals.

Discussion

Results from several cancer prevention studies (mostly *in vitro*) with cruciferous vegetables or compounds isolated from plants are contradictory, since both tumor suppression

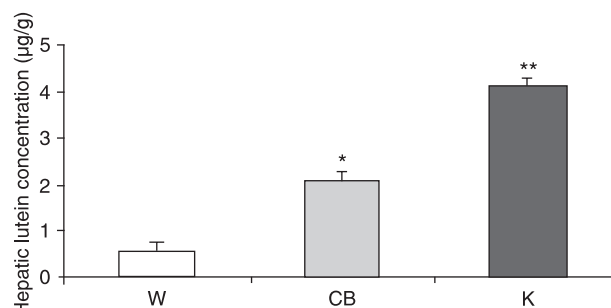


Figure 2. Hepatic lutein concentration. W = water (control); CB = cabbage; K = kale. Data are reported as means ± SD. * $P < 0.05$ compared to W and ** $P < 0.05$ compared to W and CB (ANOVA followed by the Student *t*-test).

and enhancement were reported (28,29). Treatment of rats with Brussels sprouts or red cabbage extracts at the same concentration (5% w/v) before and after IQ administration resulted in inhibition of GST-P-positive hepatic PNL, although the Brussels sprouts extract was more effective (7). This may be related to the higher concentration of glucosinolates in Brussels sprouts compared to red cabbage. These protective actions were suggested to occur during both the initiation and promotion phases of hepatocarcinogenesis. However, treatment with these same extracts specifically after IQ administration did not present inhibitory or promoting effects on hepatic PNL (8).

In the present study, we evaluated the chemopreventive potential of CB and K extracts (10% w/v) administered to Wistar rats for 8 consecutive weeks during the period comprising the initiation and promotion phases of Ito's hepatocarcinogenesis model. We used the extracts at the same concentration in order to later determine if eventual differences in chemopreventive activity could be due to differences in the content of bioactive compounds. However, in our experiment, treatment with CB or K extract did not inhibit or increase the number or aggregated area of GST-P-positive hepatic PNL. The size of GST-P PNL in rats is generally considered to be a reflection of the magnitude of hepatocarcinogenesis promotion, and their number is related to the intensity of the initiation process (30). Thus, under our experimental conditions these *Brassica* vegetables did not present chemopreventive or initiating or promoting effects at a stage that can be considered to be very early promotion, since GST-P-positive PNL were of very small size.

Several mechanisms could be responsible for the anticarcinogenic effects of *Brassica* vegetables, including induction of apoptosis (31). Smith et al. (32) showed significantly enhanced apoptosis in the colonic crypts of rats submitted to treatment with 1,2-dimethylhydrazine and Brussels sprouts. In the present study, cabbage and kale did not induce apoptosis in hepatic PNL or in the surrounding normal tissue. Considering that the level of apoptosis measurable in a target tissue can be used as a biomarker for the evaluation of compounds with chemopreventive potential (33), these results confirm the absence of inhibitory effects of cabbage and kale on hepatic PNL.

Most studies have emphasized glucosinolates and their breakdown products as the relevant compounds responsible for the biological effects of *Brassica* vegetables (34,35). However, these vegetables contain several other

bioactive compounds, including lutein (36). Cabbage and kale present lutein concentrations of 3.3 and 12.6 mg/100 g, respectively (9). The increase in hepatic lutein concentrations observed in the animals of the CB and K groups indicates effective carotenoid absorption and deposition in their livers. A higher hepatic lutein concentration in the K group than in the CB group was expected, since the K group presents higher lutein levels.

Because lutein is a carotenoid with potent antioxidant capacity (35) and oxidative stress has an important role during the initiation, promotion and progression of hepatocarcinogenesis (37), we decided to also investigate the effects of cabbage and kale on DNA strand breakage induced *ex vivo* by H₂O₂ in hepatic tissues. Ethidium bromide should be avoided in laboratory practices whenever possible. In the present study, we stained the comets with silver nitrate because of the mutagenic and carcinogenic activities of ethidium bromide, the ability to obtain a permanent record of the experiment and independent verification of the results, and also to avoid problems associated with decay of fluorescence. In addition, silver staining permits analysis of the comets with a simple light microscope instead of expensive complex equipment such as a fluorescence microscope (22,24). Both cabbage and kale presented inhibitory effects on DNA damage and these effects could be related to the observed increases in hepatic lutein levels (2 and 4 µg/g). Similarly, in rats submitted to the resistant hepatocyte model of hepatocarcinogenesis and treated with lutein, inhibitory effects on DNA damage, evaluated by the comet assay, were observed in livers with carotenoid concentrations approximately 19-25 µg/g (11,38).

Despite the absence of protective effects against PNL development and apoptosis induction, treatment with cabbage and kale resulted in increased hepatic lutein concentration and inhibition of *ex vivo*-induced DNA damage. Because the animals received the vegetable extracts during a relatively short period of time before initiation with diethylnitrosamine, the levels of lutein may be still low and insufficient to inhibit the action of the carcinogen. However, it must be pointed out that the cause-effect relationship between lutein levels and protection is hypothetical and remains to be demonstrated.

Acknowledgments

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