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ORIGINAL CONTRIBUTION

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Effect of diets fortified with tomatoes or onions with variable quercetin-glycoside content on azoxymethane-induced aberrant crypt foci in the colon of rats

Summary *Background* Onion and tomato are vegetables widely consumed by humans and epidemiological studies show an inverse association between vegetable consumption and colon cancer risk; however, the effect on colon cancer of diets containing high levels of vegetables like onion and tomato are not clear. *Aims of the study* To investigate whether tomatoes and onions, with low or high quercetin-glycoside content, could reduce azoxymethane (AOM)-induced Aberrant Crypt Foci (ACF), preneoplastic lesions in the colon of rats. *Methods* Male Fisher 344 rats were fed the following diets: a) high fat (HF) diet (control diet); b) HF diet containing 20 % (w/w) tomatoes with a low quercetin-glycoside content (final concentration in the diet: 5 mg/kg of quercetin aglycone equivalents); c) HF diet containing 20 % (w/w) high quercetin-glycoside tomatoes (100 mg/kg final concentration of quercetin aglycone equivalents); d) HF diet containing 20 % (w/w) low quercetin-glycoside onions (14 mg/kg of quercetin aglycone equivalents in the diet); e) HF diet containing 20 % (w/w) high quercetin-glycoside onions (360 mg/kg quercetin aglycone equivalents in the diet). After 2 wks of feeding, all rats were treated twice, 1 wk apart, with AOM (12 mg/kg, s. c.). The dietary treatments continued until sacrifice, 7 wks after the first injection with AOM. *Results* ACF induction did not vary in animals fed low or high quercetin-glycoside tomatoes relative to controls. On the contrary, rats fed 20 % (w/w) onion-based diets, with low or high quercetin-glycoside content, showed an increase in number, multiplicity and "large" ACF compared to the control group (number of ACF/colon 145 ± 15 (SE), 255 ± 11 and 218 ± 16 in controls, low and high-quercetin-glycoside groups, respectively; $p < 0.01$). Proliferative activity of the colon did not vary between animals fed control and high quercetin-glycoside tomato diet. The height of the crypts in normal mucosa of rats fed high quercetin-glycoside onions was significantly increased compared to control rats (cells/emicrypt 38.4 ± 1.2 (SE) and 41.3 ± 0.6 in controls and high quercetin-glycoside onions group, $p < 0.05$). *Conclusions* None of the diets supplemented with onion or tomato with variable quercetin-glycoside content demonstrated a potential chemopreventive effect on ACF-induction by AOM in rats.

Key words aberrant crypt foci – azoxymethane – onions – tomatoes – quercetin

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Introduction

Considerable attention has been focused on the identification of food products with chemopreventive activity against colon cancer, one of the major causes of death in Western countries. Epidemiological studies have shown that the consumption of diets rich in vegetables is inversely associated with colon cancer [1]. Vegetables comprise a wide range of food products of plant origin and among them tomato and onion are widely consumed by humans. Nevertheless, human studies on the

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effects of onion and tomato consumption have produced conflicting results [2–8]. While tomatoes have been associated with a decreased risk of colon cancer in some reports [2–4], they have shown no effect in others [5–6]. Similarly, onion consumption has been positively [5] and negatively [2, 6, 7] correlated with the risk of colon cancer, or with having no effect [8]. Surprisingly, the effect of tomato or onion on colon carcinogenesis in rodents has never been investigated.

Recently, new genetically modified varieties of tomatoes have been produced which contain an increased amount of quercetin-glycosides [9], one of the most abundant polyphenols present in tomatoes and onions. In addition, onion varieties high and low in quercetin-glycosides have been generated through classic breeding techniques (as reported in this paper). Although quercetin-glycosides are not the only components which may account for the beneficial effect of vegetables against colon cancer, many experimental studies suggest that polyphenolic extracts from different sources [10] or simple phenolic compounds may decrease chemically-induced colon carcinogenesis [11–13]. Given these considerations and the availability of tomato and onion varieties with increased quercetin-glycoside content, we wanted to test whether tomatoes and onions containing high levels of quercetin-glycosides were more effective in inhibiting the formation of azoxymethane-induced aberrant crypt foci (ACF) in the colon of rats than varieties containing low levels of these compounds. ACF have been purported to be the first preneoplastic lesions in the development of colon carcinogenesis [14] and have been used in short-term tests to evaluate the chemopreventive effect of many dietary components [15].

Methods

Chemicals and dietary components

AOM was purchased from Sigma Chemical (Milan, Italy). Dietary components were purchased from Piccioni Inc. (Gessate, Milan, Italy).

Preparation of tomato paste

Ripe, firm, field-grown fruit of both normal and quercetin-glycoside enriched types [9] were harvested and collected in buckets (approximately 50 kg/run) up to 16 h before processing. Fruit were rinsed in water and damaged fruit and leaves removed prior to processing. The tomato paste used in this experiment was prepared starting from just one batch of harvested tomatoes for which no attempt was made to standardise fruit maturity or size. This batch was analysed for flavonoids as described below.

Fruit was chopped and held at a break temperature of 96 °C for 5 min. The chopped fruit was pulped and the resulting material passed through a 1.5-mm screen. This purée was reduced to approximately 14 Brix using a Simulated Double Effect Evaporator at 150–180 °C, under vacuum at 558–584 mm Hg. Evaporation was continued at 304 mm Hg until a final Brix of 20–25 was achieved. The resulting paste was canned in 500 g tins, sterilised in a boiling kettle for 45 min and finally cooled under a water spray for 30 min. The preparation of non-hydrolysed flavonoid extracts was carried out as described by Krause and Galensa [16], analysing flavonoids by HPLC.

Low quercetin-glycoside tomato paste was provided by Unilever Research and contained about 47 mg/kg wet weight (w. w.) of total flavonol glycosides, corresponding to 24 mg/kg w. w. of quercetin aglycone equivalents. The flavonol glycosides were present in the following forms: 34 mg/kg (72 %) rutin, < 1 mg/kg (< 2 %) iso-quercitrin, 5 mg/kg (11 %) unidentified quercetin glycosides, 1 mg/kg (< 2 %) kaempferol-3-rutinoside, < 1 mg/kg (< 2 %) unidentified kaempferol glycosides, 7 mg/kg (15 %) quercetin trisaccharide, < 1 mg/kg (< 2 %) quercetin, < 1 mg/kg (< 2 %) kaempferol. High quercetin-glycoside tomato paste was from the same source and contained 1,376 mg/kg w. w. total flavonol glycosides, corresponding to 509 mg/kg w. w. of quercetin aglycone equivalents. The flavonol glycosides were the following: 982 mg/kg (71 %) rutin, 242 mg/kg (17.6 %) iso-quercitrin, 85 mg/kg (6.2 %) unidentified quercetin glycosides, 27 mg/kg (1.96 %) kaempferol-3-rutinoside, 20 mg/kg (1.45 %) unidentified kaempferol glycosides, 14 mg/kg (1 %) quercetin trisaccharide, 6 mg/kg (0.4 %) quercetin, < 1 mg/kg (< 0.07 %) kaempferol.

Breeding of high- and low-quercetin onion lines

As part of a Plant Research International (Wageningen, The Netherlands) onion breeding program, intra-population crosses were made between yellow and white onion lines. Segregating populations were obtained of yellow and white near-isogenic onion lines differing in flavonoid content only: yellow onions contained 28 times higher amounts of the flavonoid quercetin than white onions (492 mg versus 17 mg quercetin aglycone equivalents/kg fresh weight, respectively). Complete nutrient analysis revealed only minor differences in total reducing sugar and crude fibre content between the two types of onions (data not shown). Onions were harvested, chopped into 1 cm^2 pieces and freeze-dried. The freeze-dried onions were stored at –80 °C before use.

Yellow (high-quercetin) and white (low-quercetin) onions were provided as freeze-dried powder by Plant Research International (Wageningen, The Netherlands). Yellow onion powder contained 1,800 mg/kg of quercetin (aglycone equivalents), whereas white onion

powder contained only 68 mg/kg quercetin (aglycone equivalents). In both preparations quercetin was present as glycosides (about equal amounts of quercetin-4'-glucoside and quercetin 3,4'-diglucoside).

Animal treatments

We used 3–4 week old F344 male rats (Nossan, Correzzana, Milan, Italy). The animals were housed in plastic cages with wire tops and maintained at a temperature of 22 °C, with a 12-h light-dark cycle. The animals were treated according to the European Union Regulations on the Care and Use of Laboratory Animals [17]. The experimental protocol was approved by a local Ethical Committee for Animal Experimentation, Florence, Italy and by the Commission for Animal Experimentation of the Ministry of Health, Rome, Italy. After their arrival from the supplier, animals were quarantined for 1 week, during which they were fed standard lab chow (Teklad Global diet 18 % protein, Harlan, Correzzana, Italy). Rats were then randomly allocated in two experimental settings as described below. Experiment to test the effect of tomato: a total of 35 rats was divided into the following groups: a) controls (n = 11) fed a high fat (HF) diet, based on AIN-76 (Table 1) [18]; b) rats treated with low quercetin-glycoside tomatoes (n = 12) in which HF diet was supplemented with 20 % (w/w) tomato paste; c) rats treated with high quercetin-glycoside tomatoes (n = 12) in which HF diet was supplemented with 20 % (w/w) of tomato paste (Table 1). Experiment to test the effect of onion: rats (n = 30) were allocated in the following groups: d) a new set of controls (n = 10), fed HF diet; e) rats treated with low quercetin-glycoside onions (white onions) (n = 10), in which HF diet was supplemented with 20 % (w/w) freeze-dried onions; e) rats treated with high quercetin-glycoside onions (yellow onions) (n = 10), in which HF diet was supplemented with 20 % (w/w) freeze-dried onions (Table 2). The nutritional composition of the tomato paste and freeze-dried onions was taken into account during the preparation of the experimental diets in order to balance the diets in all groups. Diets were prepared every 2 weeks and frozen at –20 °C; aliquots were thawed at room temperature and used on alternate days. All rats were fed diet and water *ad libitum*.

To observe potential beneficial effects in any phase of the carcinogenesis process (pre-initiation, initiation and promotion phases) tomatoes and onions were fed to rats before, during and after carcinogen administration. Two weeks after the beginning of the experimental diets, rats were treated twice, 1 week apart, with AOM (12 mg/kg body weight, s. c.). Dietary treatments were continued until the sacrifice that was performed seven weeks after the first administration of AOM. Rats were sacrificed by CO_2 inhalation and colons were removed and fixed in 10 % buffered formalin.

ACF visualisation

To evaluate all ACF parameters (number, multiplicity, number of "large" ACF, defined in this study as ACF ≥ 4 crypts), colons that were fixed in formalin were stained with 0.2 % methylene blue in saline and observed under a microscope at a 40x magnification [14]. The colons were numbered with codes and scored by one observer.

Table 1 Percent of the dietary components used in the experiment to test the effect of tomatoes. *HQ* High quercetin, *LQ* Low quercetin

Components	Control Diet (g/100 g of diet)	Diet + Low quercetin-glycoside tomato paste (g/100 g of diet)	Diet + High quercetin-glycoside tomato paste (g/100 g of diet)
H_2O	15.4	/	
Casein	19.6	18.5 + 1.09 as protein supplied by 20 g of tomato paste[a]	
Corn oil	18.6	18.5 + 0.06 as fat supplied by 20 g of tomato paste	
Sucrose	30.15	28 + 2.32 as carbohydrates supplied by 20 g of tomato paste	
Starch	9.05	9.05	
Cellulose	2.5	1.6 + 0.9 as fibres supplied by 20 g of tomato paste	
AIN-76 Mineral Mix	3.64	3.2 + 0.44 as minerals supplied by 20 g of tomato paste	
AIN-76 Vitamin Mix	0.96	0.96	
Methionine	0.24	0.24	
Choline	0.16	0.16	
HQ-glycoside tomato paste			20
LQ-glycoside tomato paste		20	

[a] The diets with tomatoes contained the same amount of proteins as the control diets (i. e. 18.5 g of casein + 1.09 of proteins supplied by 20 g of tomato paste = 19.6 g of proteins). The same was true for the other nutrients present in the diet (fat, carbohydrates, fibres and minerals)

Table 2 Percent of the dietary components used in the experiments to test the effect of onions. *HQ* High quercetin, *LQ* Low quercetin

Components	Control Diet (g/100 g of diet)	Diet + Low quercetin-glycoside onions (g/100 g of diet)	Diet + High quercetin-glycoside onions (g/100 g of diet)
H_2O	2.4	/	
Casein	20.44	18.48 + 1.96 as protein supplied by 20 g of onions[b]	
Corn oil	18.52	18.48 + 0.036 as fat supplied by 20 g of onions	
Sucrose	38.95	28.4	
Starch	11.69	8.5 + 14.75 as carbohydrate supplied by 20 g of onions	
Cellulose	2.6	1.6 + 0.99 as fibres supplied by 20 g of onions	
AIN-76 Mineral mix	4.04	3.2 + 0.84 as minerals supplied by 20 g of onions	
AIN-76 Vitamin Mix	0.96	0.96	
Methionine	0.24	0.24	
Choline	0.16	0.16	
HQ-glycoside tomato paste			20
LQ-glycoside tomato paste		20	

[b] The diets with onions contained the same amount of proteins (and other nutrients) as the control diet. See note in Table 1 for details

Proliferative activity in the colonic mucosa

Proliferative activity in the colonic mucosa was evaluated using Proliferating Cell Nuclear Antigen (PCNA) immunoreactivity in slides from normal colon as described [19]. Proliferative activity was expressed as labeling index (number of labeled cells/number of the cells in the crypt section x 100). We also calculated the number of labeled cells in each of the three parts of the crypt per number of labeled cells in the entire crypt x 100. The height of the crypts was evaluated as number of cells per emicrypt. The slides were coded and read independently by two observers. The correlation coefficient between these two observers on a set of 14 labeling index scores was 0.827 ($P < 0.001$).

Statistical analysis

Data obtained from individual rats in the different dietary groups were analysed with one-way ANOVA calculating the contrasts between means using the Tukey test for multiple comparisons. Calculations were performed using the Statgraphics Statistical Package (Statistical Graphic Corporation, Rockville, MD, USA). Differences were considered statistically significant when P was < 0.05.

Results

Effect of tomato-based diets

In this experiment we assessed the effect of tomato paste in the diet (20% w/w), made from low and high quercetin-glycoside tomato varieties. The mean weight of the rats at the end of the experiment did not differ among the dietary groups (data not shown). The results of ACF determination showed that the administration of both types of tomato paste did not affect ACF number/colon (Fig.1A). Similarly, the multiplicity (number of aberrant crypt (AC)/ACF) and number of "large" ACF did not vary among groups (AC/ACF were 2.15 ± 0.05; 2.09 ± 0.06; 2.12 ± 0.05 while the number of "large" ACF was 25.5 ± 3.2; 23 ± 3.4; 24.3 ± 4 in control, low and high quercetin-glycoside tomato groups, respectively; means ± SE).

Since no difference was observed between rats fed low and high quercetin-glycoside tomato paste, we measured the proliferative activity only in the control group (n = 8) and high quercetin-glycoside tomato paste group (n = 7). The results obtained showed that the proliferative activity of normal mucosa, expressed as labeling index (LI), was not statistically different between the two groups (Fig. 1B). Besides, the distribution of labeled cells along the crypt and the height of the crypts did not vary (data not shown).

Effect of onion-based diets

We assessed the effect of onions, low and high in quercetin-glycoside on AOM-induced ACF. The mean weight of the rats at the end of the experiment did not differ among the dietary groups (data not shown). Feeding rats with low and high quercetin-glycoside onions significantly increased all the ACF parameters considered (number, multiplicity and number of "large" ACF) (Fig. 2).

We assessed proliferative activity in mucosa to test whether this effect could be explained by increased proliferation. We measured the proliferative activity only in

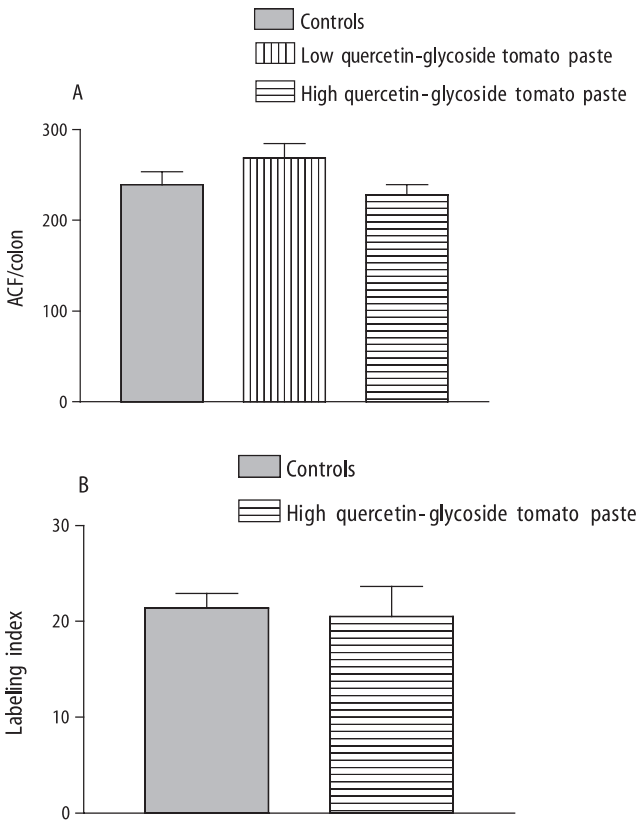

Fig. 1 **A** Number of ACF/colon. Values are means + SE (n = 11 in controls, n = 12 in low and high quercetin-glycoside groups). **B** Mucosal proliferative activity is expressed as labeling index. Values are means + SE (n = 8 in controls, n = 7 in high quercetin-glycoside group)

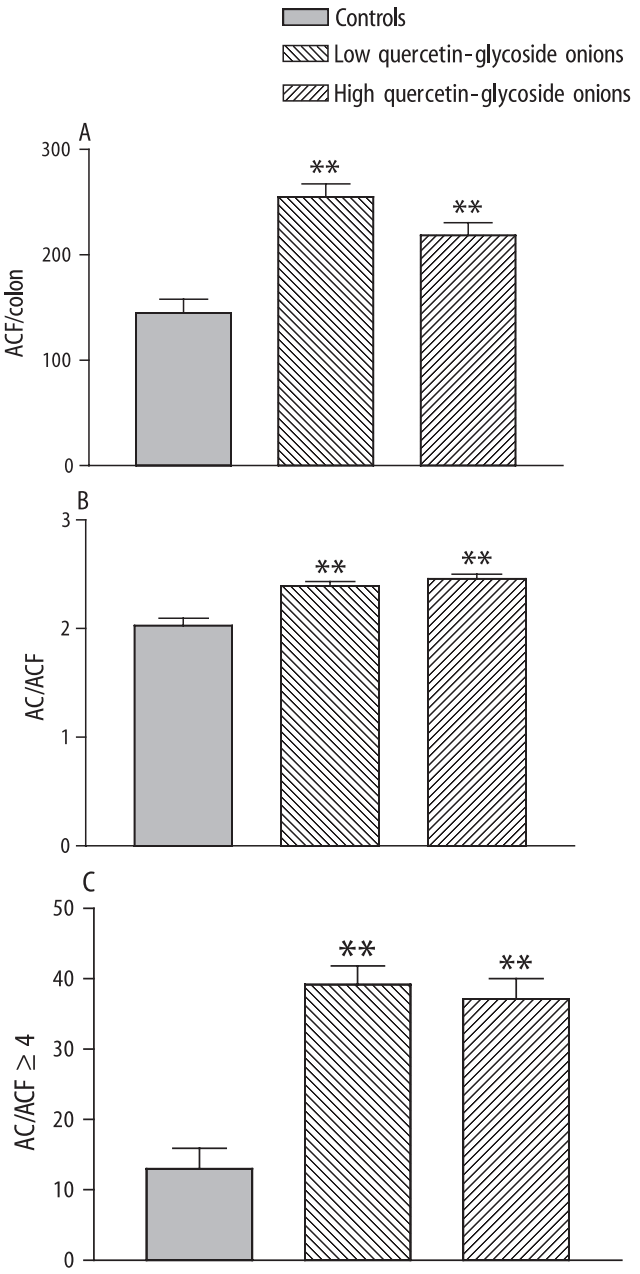

Fig. 2 **A** Number of ACF/colon. **B** Multiplicity (AC/ACF) of ACF. **C** Number of large ACF (ACF with a multiplicity equal to or larger than 4 crypts/focus). Values are means + SE (n = 10 for each group). ** = significantly different compared to the control group ($p < 0.01$)

controls (n = 6) and high quercetin-glycoside onion-fed rats (n = 8). Mucosal proliferation, expressed as labeling index (LI), was slightly, although not significantly, increased in the high quercetin-glycoside group compared to controls (Fig. 3A), but the distribution of the labeled cells along the crypt was not different (data not shown). On the contrary, the height of the crypts was significantly increased in rats fed high quercetin-glycoside onions compared to controls (Fig. 3B). We also observed that rats fed onion-based diets, with low or high quercetin-glycoside content, had significantly larger colons ($p < 0.01$) than controls (areas of the colons were 21.9 ± 1.1; 26.1 ± 1.3 and 28.1 ± 1.3 cm^2, in control, low and high quercetin-glycoside onion groups, respectively; means ± SE, n = 10 per group).

Discussion

In the present study we tested the effect of diets containing tomatoes (supplied as tomato paste) and onions (supplied as freeze-dried powder) on AOM-induced ACF in rats. We were also interested in evaluating the potential chemopreventive activity of the high quercetin-glycoside varieties of tomatoes and onions, since flavonoids are a group of naturally occurring phenols, possibly connected to the lower cancer risk of people consuming diets rich in vegetables [1]. Epidemiological studies suggest that colon cancer is strongly influenced by dietary habits, and experimental studies using rodents as the model have confirmed this observation. In

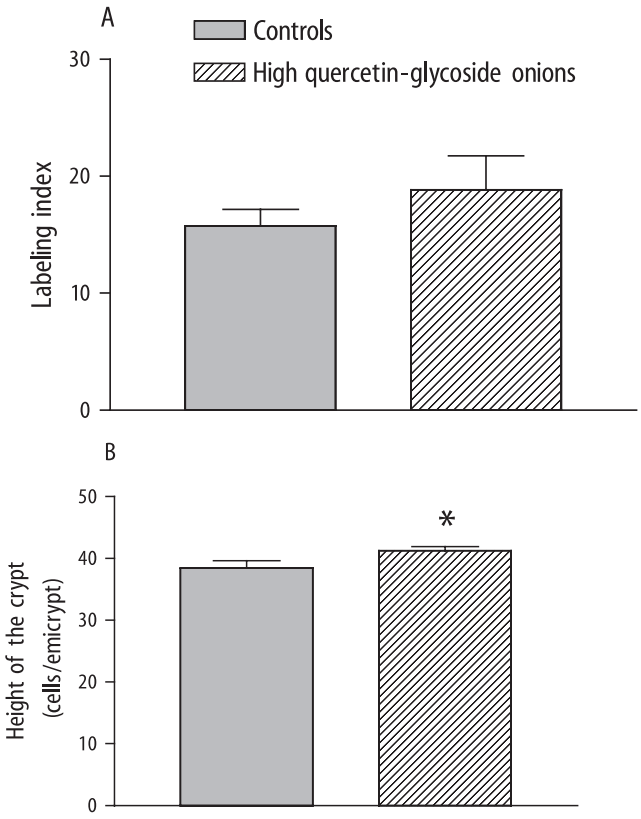

Fig. 3 **A** Proliferative activity of normal mucosa expressed as labeling index. **B** Height of the crypts (number of cells per emicrypt). Values are means + SE (n = 6 in controls, n = 8 in high quercetin-glycosides group). * = significantly different compared to the control group ($p < 0.05$)

particular, the ACF assay in which AOM-induced rats are fed with different experimental diets, has been widely used to test potentially chemopreventive agents [15]. Accordingly, it has been reported that the potency of several compounds to prevent ACF is correlated with the potency to prevent colon cancer [26].

When fed a diet containing tomato paste, rats were administered a total of about 5 or 100 mg/kg of flavonoids (mainly quercetin) in the diet (measured as aglycones) in the low and high quercetin-glycoside tomato paste groups, respectively.

Our results show that tomato paste, either low or high in quercetin-glycoside, did not reduce the number of ACF or other ACF parameters. Tomatoes contain, among other potentially active compounds, relatively high levels of lycopene, which has been previously shown to reduce AOM-induced ACF at 38 and 75 mg/kg of diet [15]. Our diets contained 56 mg/kg of lycopene in the tomato-based diet; however, they were not effective in reducing ACF. It is interesting to note that an epidemiological study was published [20] in which no association was seen between lycopene consumption and colon cancer risk. Quercetin (2% in the diet) and its glycoside rutin (4% in the diet) have been proposed to be protective against colon carcinogenesis [21, 22]. However, using a similar dose of quercetin aglycone, Pereira and coworkers observed an increase in AOM-induced tumours [23]. Using ACF induction, other authors have reported that quercetin aglycone exerts chemopreventive effects at 500 mg/kg [13] but not at 100 mg/kg [24] in the diet. Therefore, it is possible that the lack of effect observed in our experiments is due to the lower dose or possibly to the fact that we administered quercetin as glycoside and not as aglycone.

To understand if other parameters involved in the multi-step process of colonic carcinogenesis were affected by a diet rich in tomatoes, we also assessed proliferation of the normal colonic mucosa. As in the case of ACF, we did not observe a significant difference in the proliferation-related parameters evaluated.

In the study with dietary onions, low or high in quercetin-glycoside, all ACF parameters were increased compared to the control diet. Previous epidemiological studies have shown conflicting results on the effect of onion consumption on the risk of colon cancer [2, 5–7, 9]. Our diets contained a total quercetin dose (as aglycone) equal to 14 and 360 mg/kg in the low and high quercetin-glycoside onion diets, respectively, corresponding to a dose of quercetin-glycoside of 28 and 720 mg/kg, respectively. The finding that all parameters correlated to ACF were increased in this experiment suggests that, at least when present at this concentration in the diet, onions might have an enhancing effect on some steps of colon carcinogenesis.

The assessment of proliferative activity in colonic mucosa of rats from control and high quercetin-glycoside groups showed an increase in crypt height, without changes in the labeling index or in distribution of the labeled cells along the crypt. Crypt height, assessed as number of cells/emicrypt, has been used as a surrogate parameter for cell proliferation [25]. Therefore, our data suggest that dietary onions, at the concentration used, may increase proliferative activity, a conclusion also supported by the observation that rats fed onion-based diets have an increased colonic size.

We cannot rule out the possibility that a lower onion supplementation may have had a beneficial effect or that a possible beneficial effect of flavonoids was masked by the influence of an excessively high content of onions in the experimental diet. Moreover, as in the previous study on tomatoes, it is important to note that quercetin-glycosides may affect the colon or other biological targets in a different manner when compared to quercetin aglycone.

In conclusion, none of the experimental diets supplemented with tomatoes or onions, low or high in quercetin-glycosides, had a suppressive action on ACF induction by AOM. On the contrary, onions added to the diet at high concentration showed an enhancing effect

on some steps of colon carcinogenesis, as indicated by an increase in ACF parameters. Therefore, none of the supplements tested, at least at the concentrations used, seem to have chemopreventive potential.

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