

## RESEARCH ARTICLE

# Grapefruit Juice Suppresses Azoxymethane-induced Colon Aberrant Crypt Formation and Induces Antioxidant Capacity in Mice

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### Abstract

In the present report we determined the protective capacity of grapefruit juice (GJ) against molecular and cellular damage in azoxymethane (AOM) treated mice. Animals were daily administered GJ orally (0.8, 4.1, and 8.2  $\mu$ l/g) for seven weeks, as well as intraperitoneally (ip) injected with AOM twice (weeks 2 and 3 of the assay). Control groups administered with water, with the high dose of GJ, and with AOM injected in weeks 2 and 3 were also included. The results showed a significant, dose-dependent protection of GJ on the number of colon aberrant crypts (AC) induced by AOM. The highest inhibitory effect was reached with the highest tested dose of GJ, decreasing ACF by 51% and 43% at weeks 4 and 7 of the assay. Regarding protein and lipid oxidation we also found a dose-dependent decrease caused with GJ in comparison with the increased levels produced by AOM. Therefore, our results established chemopreventive potential for GJ, and suggested effects related to its antioxidant capacity. Finally, we found that the tested agents induced neither micronuclei increase nor alteration in bone marrow cytotoxicity.

**Keywords:** Grapefruit juice - aberrant crypts - chemoprevention mechanism - antioxidant capacity

*Asian Pac J Cancer Prev*, 14 (11), 6851-6856

### Introduction

Cancer is becoming a relevant public health problem in most countries, in such form, that by the end of this decade it has been suggested that one of each of four deaths will be related with the disease, although with geographical variations, which may be influenced by interactions among age, environment, and the genetic background of the affected individuals (Smart et al., 2008; WHO, 2013). The colorectal cancer in particular is also highly present and may represent the third most common type of cancer in western countries (Ferlay et al., 2010). This high frequency, as well as the increasing knowledge about the participation of the diet and lifestyle in its development, which is concordant with the continuous information on the importance of preventive measures to control the disease, has promoted the search of chemopreventive agents that can be used to reduce the incidence of this type of cancer. An approach which is related with the identification and validation of three distinct classes of agents: those that can be inhibitors of carcinogen formation, agents with can be inhibitors of tumor initiation, and agents that can suppress tumor promotion and/or progression (Stoner et al., 1997; Namasivayam, 2011).

Grapefruit is a plant whose fruits are widely consumed

in natural or in different commercialized food products, and that possess a number of nutritive constituents; moreover, consumption of the plant has been related with various biomedical effects, including antimicrobial effects, as well as beneficial properties on diverse aspects of obesity, diabetes, and cardiovascular disease (Adeneye, 2008; Diaz-Juarez et al., 2009).

Besides, under the light of the intimate relationship between mutagenesis and carcinogenesis, the reported capacity of the plant for reducing genetic damage is of particular importance for the present study. In this field, various reports with grapefruit juice (GJ) both *in vivo* and *in vitro* have shown a clear antigenotoxic effect on the DNA and chromosomes damaged by mutagens with different mechanisms of action (Alvarez-González et al., 2004; Miyata et al., 2004; Razo-Aguilera et al., 2011). Besides, in the area of carcinogenesis, *in vitro* studies conducted on MCF-7 human breast cancer cells, or in Caco-2 and HL-60 cells support the idea that components of grapefruit (limonin and naringenin) have apoptotic activity that limits the growing of cancerous cells; also, the capacity to inhibit the development of cancer by grapefruit or because the activity of specific constituents have also been suggested in *in vitro* studies, as well as at the *in vivo* initiation or post-initiation stages (So et al., 1996; Tian et

al., 2001; Kanno et al., 2005; Vanamala et al., 2006; Miller et al., 2008; Hwang et al., 2010). The findings obtained in these studies are in close agreement with the general suggestion that the consumption of fruits and vegetables reduces the risk of colon cancer (Koushik et al., 2007).

On the other hand, a number of reports have established the strong antioxidant potential of GJ, as well as of several of its components (Alvarez-Gonzalez et al., 2004; Gorinstein et al., 2005). Moreover, this effect has been suggested to be, at least partially, the subjacent explanation for the observed antigenotoxicity of the juice. In this context, it is known that azoxymethane (AOM) is a specific colon carcinogen that has been extensively used to experimentally study the carcinogenesis/anticarcinogenesis processes. AOM is a DNA alkylating chemical able to induce aberrant crypts (AC), putative precancerous lesions in the colon of experimental rodents which are considered surrogate biomarkers useful in screening potential chemopreventive, as well as carcinogenic compounds in the colon (Bird, 1995; Bird and Good, 2000). Therefore, according to the previously shown information, the first aim of the present report was to evaluate whether the administration of GJ to AOM-treated mice inhibits the amount of AC. Besides, it is also known that oxygen and hydroxyl radicals, as well as hydrogen peroxide play a significant role in the pathogenesis of colon cancer, and that AOM is able to induce such an effect, for example, by increasing the lipoperoxidation and iNOS levels, as well as by reducing the level of glutation (Karabulut et al., 2009); therefore, the second aim of the report was to evaluate whether the anti-crypt effect of the juice could be related with its antioxidant potential; finally, it has been reported that a number of mutations are produced during the colon carcinogenesis, including those in K-ras, APC, and p53 genes, as well as different chromosome gains and losses (Lea et al., 2009); therefore, we also explored whether these damages could be expressed as an increase in the number of micronuclei.

## Materials and Methods

### *Chemicals and animals*

The following chemicals were purchased from Sigma Chemicals (St Louis Mo USA): AOM, trichloroacetic acid (TCA), ethanol- ethyl acetate, tris-HCl, guanidine, albumin standar, tiobarbituric acid (TBA), methylene blue, and 2,4-dinitrophenylhydrazine (DNPH). Formaldehyde, PBS, HCl, methanol and ethanol were acquired from Fermont (Mexico City), and the Giemsa stain was obtained from Merck (Mexico City). Grapefruits (*Citrus paradisi* Mcfad var Ruby red) were obtained from a pesticide free cultivar in Veracruz, México, and the juice was freshly prepared immediately before its administration.

The experimental protocol with animals was approved by the Committee of Ethics and Biosecurity of the National School of Biological Sciences. For the research we used CF-1 female mice with a mean weight of 25g. The animals were maintained in metallic cages at 23°C, 40% humidity, and in a 12h dark-light cycle. Mice were permitted to freely consume nutricubes (Rodent Laboratory Chow

5001, Purina), and water.

### *Experimental design*

Six groups with 16 animals each were organized and treated as follows along the seven weeks of the study: one group was daily administered with 0.3ml of drinking water by the oral route, a second group was daily administered with 8.2µl/g of GJ, orally, a third group (positive control) was intraperitoneally (ip) injected 10mg/kg of AOM in the second and third week of the assay only (twice each week), and three more groups were daily administered by the oral route with 0.8, 4.1, and 8.2µl/g of GJ during the 7 weeks of the study, as well as with an ip injection of 10mg/kg of AOM, twice a week, only in the second and third week of the assay. The selected doses of GJ were based in previous reports (Alvarez-Gonzalez et al., 2011; Argüelles et al., 2012) as well as in preliminary assays which considered the present exposure time. From the low to the high dose they were equivalent to the ingestion of a 50ml glass of juice by a 60kg person, and to one or two glasses of 250ml, respectively. The weight and the food consumption of mice was registered every third day along the assay.

### *Quantification of colon crypts*

Eight mice of each group were cervically dislocated at the four week of treatment and the other eight at the end of week seven. Then, the colon was obtained, washed in PBS, longitudinally open, and fixed for 24h in 10% formaldehyde made in PBS 0.1M, at pH 7.4 and at 4°C. Finally, the tissue was stained with 0.2% methylene blue for 30min. The number of crypts was determined at 40X magnification (Carl Zeiss microscope, Germany). The presence of crypts was recognized from surrounding cells by their increased size, elongated luminal opening, increased distance from the luminal to basal surface of cells, thickened epithelial cell lining, and enlarged pericryptal area relative to surrounding normal crypts (Bird, 1995; Murillo et al., 2004). Single and multiple crypts were registered.

### *Quantification of total and oxidized proteins*

A small portion of colon was homogenized with PBS pH 7.4 in 1:4 relationship, and the soluble proteins were obtained by a centrifugation at 10000g for 15min. Then, we quantified the amount of total proteins by the method of Bradford (1976). For the calculation we interpolated the obtained absorbance values in a calibration curve made with albumin standar constructed with 0, 10, 25, 50, 75, and 100mg/ml of the substance. The quantification of proteins and that of the curve type was made by triplicate.

To determine the level of protein oxidation we used the method of Levine et al. (1994). We added 1ml of 20% cold TCA to each sample of soluble proteins and made a centrifugation at 13000g for 15min, then, we added a mix of 100ml PBS plus 100ml of DNPH diluted in HCl 2M. In parallel, we also prepared HCl 2M as blank, and the substances were incubated at 37°C for 1 h. After that time, 500µl of a 20% solution of TCA was added and the precipitated protein was collected by centrifugation at 1000g for 10 min at 4°C. The precipitate was washed with

500 $\mu$ l of a 1:1 mix of ethanol- ethyl acetate, and dissolved in 1ml of guanidine 6M made in potassium phosphate buffer 2mM at pH 2.3. The samples were incubated at 37°C for 40 min and the absorbance was measured at 360nm. The carbonyl content was expressed in nM of DNPH formed by mg of protein. We considered 21000 M<sup>-1</sup>cm<sup>-1</sup> as the molar absorptivity value.

#### Lipid peroxidation

For this assay, the technique described by of Buege and Aust (1978) was applied. We added to the soluble protein sample 700 $\mu$ l of the buffer tris-HCl 50mM, pH 7.4 plus 2ml of a 0.0375% solution of TBA made in 15% TCA. The samples were incubated at 37°C for 45min, and centrifuged at 3000g for 5min. Absorbance was determined at 535nm, and the results expressed in mM of malondialdehyde/mg protein/g of tissue. Molar absorptivity was considered 1.56 $\times$ 10<sup>5</sup> M<sup>-1</sup>cm<sup>-1</sup>.

#### Micronuclei and cytotoxic determination

Blood samples from the tail of each mouse were taken before the treatment and at the end of each of the seven weeks of the assay. They were smeared in clean slides, fixed with methanol for 3 min, and stained with Giemsa solution made in PBS for 12 min. For the genotoxic/antigenotoxic study we registered the number of micronucleated normochromatic erythrocytes (MNNE) in 2000 normochromatic erythrocytes per mouse. For the determination of bone marrow cytotoxicity we registered the proportion of polychromatic erythrocytes respect to the number of normochromatic erythrocytes in 2000 cells per mouse.

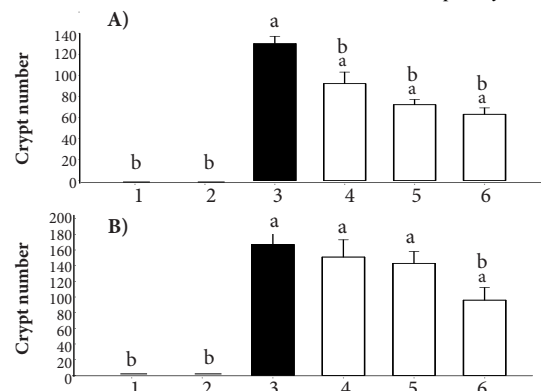
#### Statistical analysis

The statistical evaluation was made by applying the ANOVA test and the Student-Newman-Keuls test, by means of the Sigma Stat Software, version 2.03.

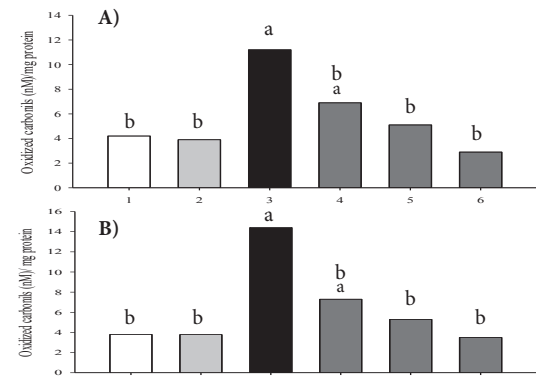
## Results

With respect to the weight we found no significant modifications in the studied groups of animals. The mean value for all groups before the chemical administration was 25.38 $\pm$ 1.9 g, and at the end of the assay the mean weight was 27.7 $\pm$ 0.6. The mean daily food consumption along the experiment was 3.6 $\pm$ 0.3g. No statistical differences were found among groups.

The results concerning the number of colon crypts are shown in Figure 1. The upper part (Figure 1A) refers to data obtained at week 4 of the study. At this time we detected crypts nor in the water-administered mice neither in those treated with 8.2 $\mu$ l/g of GJ; in contrast, animals administered with AOM reached 130 crypts. When GJ was administered along the assay together with AOM administered in weeks 2 and 3, the result indicated a dose-dependent decrease in the number of AC with respect to the result obtained in the AOM treated mice; the highest reduction (51%) was found with the high tested dose (8.2 $\mu$ l/g), followed by the lower doses of 4.1 and 0.8 $\mu$ l/g 20 of GJ which showed an inhibition of 45% and 29%, respectively. At week 7 the results were similar for the



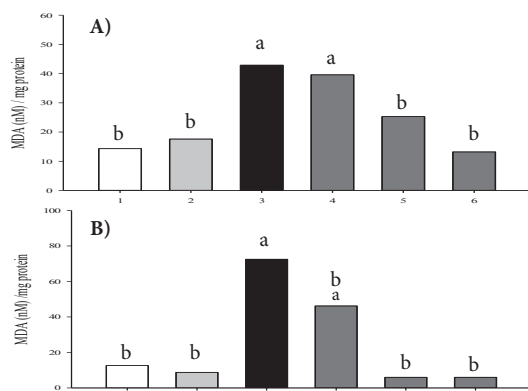
**Figure 1. Aberrant Crypt Foci in the Colon of Mice Treated with Azoxy methane (AOM) and Grapefruit Juice (GJ).** 1=Control group (water), 2=GJ (8.2 $\mu$ l/g) 3=AOM (10mg/kg) 4=GJ (0.8 $\mu$ l/g)+AOM (10 mg/kg) 5=GJ (4.18.2 $\mu$ l/g)+AOM (10mg/kg) 6=GJ (8.2 $\mu$ l/g)+AOM (10mg/kg), (A) Results obtained at 4 weeks of treatment, and (B) at 7 weeks. Each bar represents the mean $\pm$ SEM obtained in the colon of 8 mice per treatment. <sup>a</sup>Statistically significant difference with respect to the control value, and <sup>b</sup>with respect to the group treated with AOM. ANOVA and Student-Newman Keuls,  $p \leq 0.05$ .



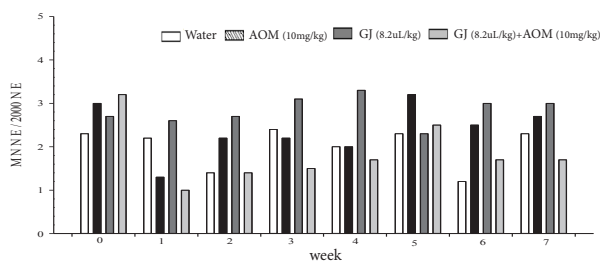
**Figure 2. Induction of Oxidized Protein in the Colon of Mice Treated with Azoxy methane (AOM) and Grapefruit Juice (GJ).** 1=Control group (water), 2=GJ (8.2 $\mu$ l/g) 3=AOM (10mg/kg) 4=GJ (0.8 $\mu$ l/g)+AOM (10mg/kg) 5=GJ (4.18.2 $\mu$ l/g)+AOM (10mg/kg) 6=GJ (8.2 $\mu$ l/g)+AOM (10mg/kg), (A) Results obtained at 4 weeks of treatment, and (B) at 7 weeks. Each bar represents the mean $\pm$ SEM obtained in the colon of 8 mice per treatment. <sup>a</sup>Statistically significant difference with respect to the control value, and <sup>b</sup>with respect to the group treated with AOM. ANOVA and Student-Newman Keuls,  $p \leq 0.05$ .

control and the GJ treated animals which showed no crypts; also, an elevated number of crypts was observed with the administration of AOM (167.7 $\pm$ 15), and a dose-dependent decrease of such lesions was determined with the three tested doses of GJ when they were administered in AOM treated mice, showing an inhibition of 43, 21, and 9% respect to the value obtained with the carcinogen, only. In this case, however, significance was reached only with the high tested dose of the juice. In regard to multiple crypts, we found numbers which varied from 2-10, being the more frequent range from 2-5 crypts. Their distribution among groups was generally homogeneous.

Figure 2 refers to the results obtained regarding the level of oxidized proteins. We found that mice administered only GJ had values of reactive carbonyls which were similar to the observed in the control animals



**Figure 3. Lipid Peroxidation Induced by Azoxymetane (AOM) and Grapefruit Juice (GJ) in the Colon of in Mouse.** 1=Control group (water), 2=GJ (8.2 μl/g) 3=AOM (10mg/kg) 4=GJ (0.8 μl/g)+AOM (10 mg/kg) 5=GJ (4.18 μl/g) +AOM (10mg/kg) 6=GJ (8.2 μl/g)+AOM (10mg/kg), (A) Results obtained at 4 weeks of treatment, and (B) at 7 weeks. Each bar represents the mean±SEM obtained in the colon of 8 mice per treatment. a Statistically significant difference with respect to the control value, and b with respect to the group treated with AOM. ANOVA and Student-Newman Keuls,  $p \leq 0.05$



**Figure 4. Induction of Micronucleated Normochromatic Erythrocytes (MNNE) by Azoxymetane (AOM) and Grapefruit Juice (GJ) in Mouse Peripheral Blood.** Bars correspond to the mean±SEM obtained in 2000 normochromatic erythrocytes per sample. No statistical difference was observed. ANOVA and Student-Newman Keuls,  $p \leq 0.05$

along the assay; however, mice administered AOM showed an increase of 2.8 times in comparison with result of the control group at week 4 of the assay, and of 4.2 times of increase at week 7. On the other hand, GJ prevented the protein oxidation in a dose-dependent manner. At week 4 the inhibition was of 36, 54, and 100%, with the addition of 0.8, 4.1, and 8.2 μl/g of juice, and at week 7 the protection was of 49, 63, and 100%, with the above mentioned doses.

The lipid peroxidation effect of the tested agents was shown by the generated MDA (Figure 3). We found no increase in the MDA level with the exposure to GJ either at the fourth or the seventh week of treatment, however, mice administered with AOM had an elevation of three times and of more than five times the level of MDA at week 4 and 7, respectively, as compared with the control value. When animals were treated with both agents (GJ and AOM), the doses of 8.2, 4.1, and 0.8 μl/g of GJ induced a dose-dependent reduction of the level of lipid peroxidation in comparison with AOM-treated animals. At week 4, the effect obtained with 8.2 μl/g of GJ gave rise to a level similar to the observed in the control group, and at week 7 such similarity was obtained with 0.8 and

8.2 μl/g of the juice.

In regard to the determination of micronuclei the obtained results are shown in Figure 4, which indicates that the high tested dose of GJ was not genotoxic per se along the treatment, as the weekly MNNE mean obtained in such group was similar to the determined in the control animals. A similar result was determined in animals treated with the combination of both agents. However, the protective effect of the juice could not be evaluated as we also found no micronuclei increase by AOM in the present experimental conditions. The bone marrow cytotoxicity results are shown in Figure 5. The obtained results are congruent with the above mentioned data because the mean proportion PE/NE for the GJ and the AOM administered animals were in the same range as those of the control group.

## Discussion

The study of AC for analyzing colon carcinogenesis has been validated in mouse and rat as well. A number of evidences support the conclusion that AC constitute initial lesions that may give rise to adenomas and carcinomas in advanced steps (Takayama et al., 2005). The usefulness of evaluating these lesions also relies on the shorter time of experimentation compared with the classical methods for studying tumor development, as well as in the similarity of AC in experimental animals as compared with the observed in humans (Pretlow et al., 1991). In the present report we confirmed the development of a high number of AC by the action of AOM at the two evaluated times, indicating that experimental approaches may give appropriate data at a varying times after the AOM administration, which goes from three to ten or fifteen weeks after the carcinogen injection, however, the progression of multiple crypt clusters seems to be better observed at delayed times. Our results also established the significant protection exerted by GJ when administered at the initiation phase. The juice could have interfered with initial genetic and/or oxidative carcinogen-induced events of the process. This effect is congruent with the observed by Vanamala et al. (2006), who pre-administered grapefruit pulp powder for longer time than in our case, to rats injected with 15 mg/kg of AOM. Our result also agree with the decrease in colon crypts reported by the activity of constituents of the juice, such as naringenin, naringin, quercetin, rutin, kaempferol, and lycopene (Wargovich et al., 2000; Vanamala et al., 2006; Warren et al., 2009; Leonardi et al., 2010; Nirmala and Ramanathan, 2011a). These results suggest that the observed precarcinogenic prevention by GJ could be related with a synergistic effect exerted by several of its components. Moreover, these results may also suggest that the effect can be related with various mechanisms of action. In several of the above mentioned chemopreventive studies with grapefruit or its components, authors have observed modifications on activities of compounds such as phosphatidylinositol 3-kinase, or the proinflammatory mediators cyclooxygenase (COX-1 and COX-2), as well as on the inducible nitric oxide synthase proteins. In some cases, these determinations were positively correlated with the capacity of the tested chemopreventive agent for

inhibiting proliferation and accelerating apoptosis.

Chemical carcinogenesis of the colon is a multistep process where persistent oxidative stress may lead to DNA damage, mutation of cancer related genes, besides the epigenetic silencing of tumor suppressor genes, proliferative increase, inflammation and apoptotic tissue damage (Klaunig and Kamendulis, 2004; Khan and Sultana, 2011; Nirmala and Ramanathan, 2011a). In the present report, we demonstrated a significant reduction by GJ on the level of lipid and protein oxidation induced by AOM, suggesting that the antioxidant capacity of the juice is involved in the carcinogenic prevention. Moreover, the inhibition of DNA oxidation by GJ on mutagens and teratogens has also been determined *in vitro* and *in vivo*; in this respect, oxidized guanine residues in hydrogen peroxide-treated human lymphocytes were lowered by the juice as shown by adding the enzyme formamidopyrimidine-DNA-glycosylase to the comet assay, similarly, it was reported a reduction in the amount of the adduct 8-hydroxy-2'-deoxyguanosine in the serum of cadmium-treated fetuses (Razo-Aguilera et al., 2011; Argüelles et al., 2012). In fact, the antioxidant potential of GJ, as well as of various constituents has been clearly established in a number of tests, although only two of them has been carried out *ex vivo* (Alvarez-González et al., 2004; Gorinstein et al., 2005; Sengupta et al., 2006; Ademosun and Oboh, 2012). Also, a relationship between antioxidation and colon crypt inhibition has been documented in studies measuring lipoperoxidation, as well as enzymatic and non-enzymatic compounds (Nirmala and Ramanathan 2011a; 2011b; Khan and Sultana, 2011). These data clearly reveal that agents reducing oxidative stress are relevant for impeding the cascade of events in colon carcinogenesis.

The acquisition of genomic instability is a crucial feature in tumor development; in colon pathogenesis at least three distinct pathways are known, including microsatellite instability, CpG island methylator phenotypes, and chromosomal instability which can result in chromosome number imbalances and loss of heterozygosity (Pino and Chung, 2010; Migliore et al., 2011). However, in our assay we found no increase in the number of micronuclei in the analyzed blood cells. This result agrees with the fact that the initial genetic damage by the carcinogen could be organ-specific, is of epigenetic type, or/and that defects in chromosomal segregation are produced later in the pathogenesis, probably during the adenoma-carcinoma phase. In fact, the molecular mechanism that act at the colon cancer initiation are complex and not homogeneous, for example, K-ras mutations can be present in most sporadic AC, in contrast with adenomatous polyposis where APC mutations are preponderant (Takayama et al., 2005).

In summary, in the present assay we demonstrated a chemopreventive potential for GJ, probably related with its antioxidant capacity; a result that adds new information to the described beneficial effects of the juice, such as its antigenotoxic and antiteratogenic capacities, among others, The obtained results may be valuable in light of the common use of grapefruit and its juice in human diet; however, before considering a chemopreventive candidate

other studies can be done, for example, concerning other mechanisms of action, the more appropriate doses particularly in regard to its interaction with medicaments, as well as the weight of its particular components in the effect.

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