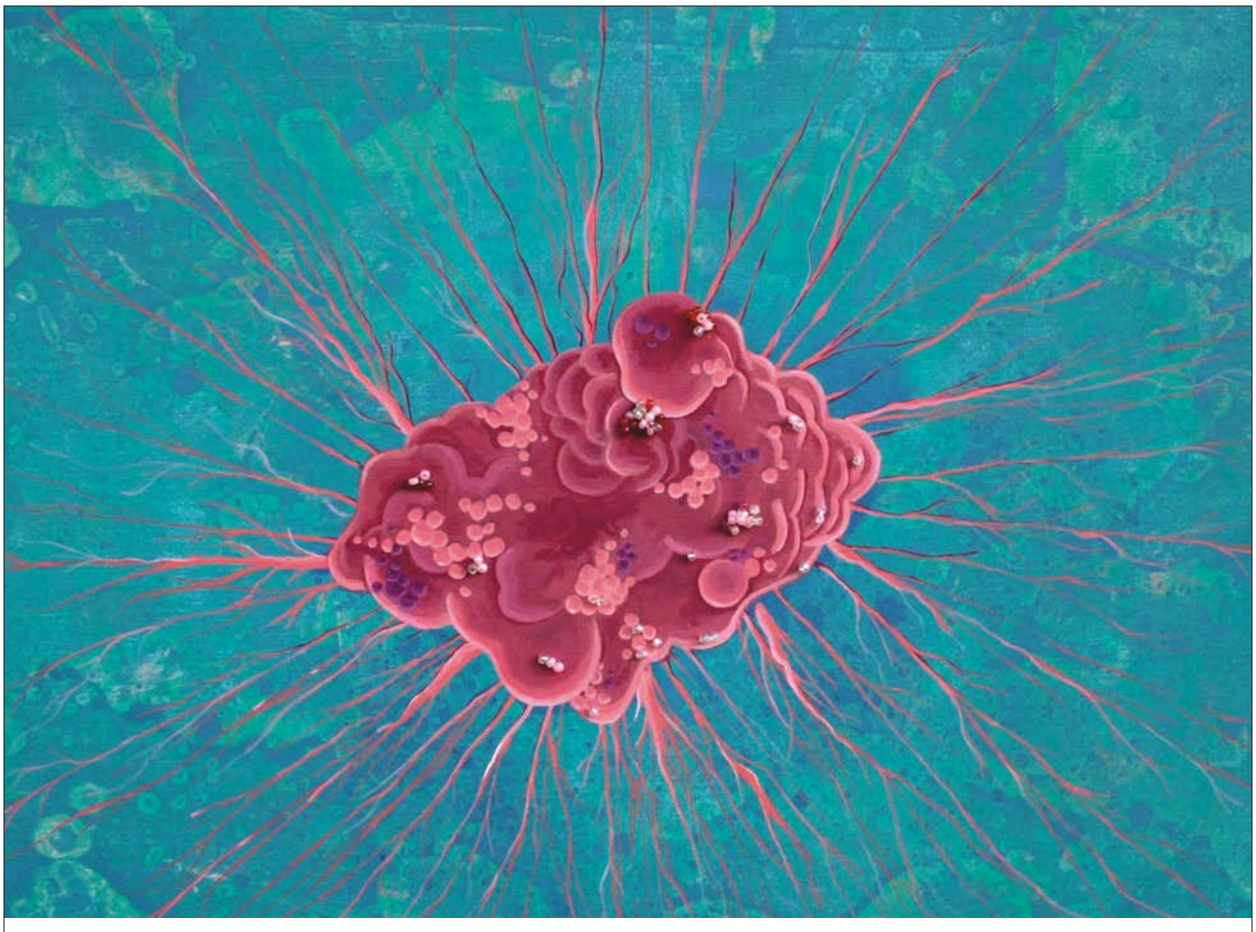

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Grape Products Reduce Colon Cancer in Azoxymethane-induced Aberrant Crypt Foci in Fisher 344 Rats

J. Jones, M. Verghese, L.T. Walker, L. Shackelford and C.B. Chawan
Department of Food and Animal Sciences, Nutrition and Carcinogenesis Laboratory, Alabama A and M University, P.O. Box 1628, Normal, AL, 35762, United State of America

Corresponding Author: M. Verghese, Nutrition and Carcinogenesis Laboratory, Department of Food and Animal Sciences, Alabama A and M University, P.O. Box 1628, Normal, AL, 35762, United State of America Tel: (256) 372-5454 Fax: (256) 372-5432

ABSTRACT

Inhibitory potential of grape products on Azoxymethane (AOM) induced Aberrant Crypt Foci (ACF) were investigated in Fisher 344 male rats. Bioactive components present in the grapes have shown anticarcinogenic properties in *in vitro* and epidemiological studies. Following an acclimatization period of one week, 24 male weanling rats were fed control and experimental diets consisted of either grape Juice (50%) or Raisins (10%). All rats received 16 mg kg^{-1} b.wt. of AOM at 7 and 8 week of age. Rats were euthanized by CO_2 at 17 week of age. Number of ACF was enumerated in colon and Glutathione S-transferase (GST) activity was analyzed in liver of rats. No significant differences were observed in the weight gain of rats among the groups. Highest (73%) reduction in ACF was found in rats fed with Grape juice followed by rats fed with raisins (66%). The total number of crypts were significantly lower in treatment group rats (Juice-93.62±4.3; raisins-116.66±5.3) compared to the control group (358±13.3). Glutathione S-transferase enzyme activity was increased by 2-3 fold in the treatment groups. This study indicates that administering grape juice at 50% and raisins at 10% in diet of rats significantly reduced the ACF in Fisher 344 male rats and may offer protection against colon cancer development.

Key words: Colon cancer, aberrant crypt foci, grapes, resveratrol, glutathione S-tranferase

INTRODUCTION

Colorectal Cancers (CRC) develop slowly over a period of several years. Previous to tumor development, it usually begins as a polyp, which may eventually become cancerous. If not treated, cells may separate from the tumor and spread through the bloodstream or lymph system to other parts of the body (ACS, 2005). The United States, colorectal cancer is one of the most frequently diagnosed cancers after lung, breast and prostate cancers (ACS, 2012). It is also the second most common cause of death from cancer in the United States. According to the American Cancer Society (ACS), an estimated 143,460 cases will be diagnosed in 2012 in conjunction with 51,690 deaths (ACS, 2012). Although medical techniques have highly developed, there has been only a minimal amount of progress in survival for patients who have developed advanced stages in cancer. Aberrant Crypt Foci (ACF) are recognizable precursors to colon cancer in carcinogen-treated rat colons (Bird, 1987). The morphological and genotypic features of ACF in human colons are similar to those in animal colons and many characteristic variations in ACF are similar in tumors; ACF

display similar innate variations and histological changes often seen in human colonic lesions (Stopera *et al.*, 1992; Losi *et al.*, 1996). These precancerous lesions can be induced by colon carcinogens (McLellan *et al.*, 1991) and transformed by certain inhibitors of carcinogenesis (Wargovich *et al.*, 1992). ACF provide a simple and efficient method for early screening of potential chemo preventive agents and it allows for a quantitative evaluation of the mechanisms of colon carcinogenesis.

Researchers around the world have focused on developing alternative approaches to prevent colon cancer. Flavonoids are a naturally produced plant based chemicals that can be found in substances that contain a phenolic structure such as: fruits, vegetables, bark, grains, roots, tea, flowers, stems and wine. Protective effects of these natural compounds were known from folk medicine and later beneficial effects were attributed to flavonoids present in them (Nijveldt *et al.*, 2001). Another essential effect of flavonoids is the capability to scavenge oxygen-derived free radicals. Experiments (*in vitro*) have demonstrated that flavonoids possess key activity such as: anti-inflammatory, antiallergic, antiviral and anticarcinogenic properties (Nijveldt *et al.*, 2001). Resveratrol (trans-3,4', 5-trihydroxystilbene) is a naturally produced plant metabolite with medicinal qualities (Kimura and Okuda, 2001). It offers protection against cardiovascular diseases and chemically induced cancers. Reports have illustrated that resveratrol inhibits tumor growth and induces apoptosis as a cancer chemopreventive mechanism (Kimura and Okuda, 2001).

Colorectal tumors are extremely frequent in western populations. Chemopreventive agents present in the diet offer great potential in reducing the incidence of cancer (Wolter and Stein, 2002). Recent studies have shown that there is a correlation between the reduced risk of colorectal cancer and diets high in fruit, fiber or vegetables (Wolter and Stein, 2002). An important biomarker of carcinogenesis is the increased activity of the detoxification enzyme glutathione S-tranferase. Glutathione S-tranferases (GSTs) are a group dimeric proteins which principal function is to detoxify electrophiles capable of binding DNA during Phase II metabolism (Pickett and Lu, 1989). The objective of this study was to observe the anticarcinogenic effect of grape products (Concord grape juice (CGJ 50%) and raisins-10%) on azoxymethane-induced aberrant crypt foci (preneoplastic lesions) in Fisher 344 male weanling rats and to demonstrate their effect on the activity of glutathione S-transferase (a key phase II detoxification enzyme) in the liver.

MATERIALS AND METHODS

All chemicals, including azoxymethane, were obtained from Sigma Chemical Company. Dietary ingredients were obtained from ICN (Costa Mesa, CA).

Animals, housing and diets: Male Fisher 344 weanling rats were obtained from Harlan, IN and housed in stainless steel wire cages at two rats per cage. The temperature and relative humidity were maintained at 21°C and 50%, respectively. Light and dark cycles were scheduled at 12 h each. Feed and water were provided *ad libitum*. After a one-week period of acclimatization, the animals were randomly divided into groups and fed the experimental diets until 16 weeks of age. During this time, biweekly body weights and daily feed intakes were recorded. All rats received American Institute of Nutrition (AIN) 93G based diets.

Carcinogen injection: For induction of colon tumors, all animals received a subcutaneous injection of Azoxymethane (AOM) in saline at 16 mg kg^{-1} b.wt. one dose at 7 weeks and another at 8 weeks of age.

Table 1: Control and experimental diets

	Grape products (g kg^{-1})		
Ingredients	Control	Raisins (10%)	Grape juice (50%)
Corn starch	397.5	397.5	397.50
Alphacel	50	46.8	50.00
Dextrose	132	108.8	132.00
Sucrose	100	76.8	91.05
Grape products	0	100.0	0.00
Common ingredients[1]	320.5	320.5	320.50

[1]Common ingredients: Casein (>85% protein), 200; soybean oil (no additive), 70; AIN-93M mineral mix, 35; AIN-93G vitamin mix, 10; L-cysteine, 3; choline bitartrate (41.1% choline), 2.5

Diets: AIN 93 G Control (C) diets were prepared fresh at intervals of 4 weeks or less and stored at refrigeration temperature (~4°C). Concord Grape juice: CGJ (Welch's, MA) was prepared at 50% concentration and administered fresh daily. Administered with CGJ was AIN 93 control diet with alterations in the level of sucrose. Raisins (Sunmaid, CA) were incorporated at a 10% level in the diet with appropriate modifications in dextrose, sucrose and alphacel (fiber) (Table 1).

Colon sample collection: At 17 week of age, the rats were euthanized using CO_2 asphyxiation after an overnight fasting. Colons of the rats were removed and flushed with potassium phosphate buffer (0.1M, pH 7.2) and then analyzed for Aberrant Crypt Foci (ACF) (Bird, 1987).

Counting the ACF: Colons were split open longitudinally and positioned on filter paper with the luminal surface open and exposed. A second filter paper was placed on top of the luminal surface directly onto the colon. As a result, the colons were secured and fixed overnight using 10% buffered formalin. Each fixed colon was cut into proximal and distal portions of equal length and each portion was further cut into 2 cm long segments. Each segment was placed in a petri dish and stained for 5 min using 0.5% methylene blue solution. The segments were transferred to another petri dish containing buffer to wash the excess stain for further examination under a light microscope to score the total number of ACF, as well as the number of crypts per focus as described by Bird (1987).

Glutathione-S-transferase (GST) assay: GST activity in the liver was assayed by the procedure of Habig *et al.* (1974). Liver samples were homogenized in 10 volumes of potassium phosphate buffer (pH 7.0, 0.1 M) in Potter-Elvejem homogenizer at 4°C. The homogenate was centrifuged at 10,000 g for 30 min. The assay mixture (1 mL) contained potassium phosphate buffer (0.1 M, pH 6.5), 1, Chloro 2,4-dinitrobenzene (1 mM) and glutathione (1 mM). Reactions were started by the addition of 50-100 µL of sample and change in absorbance at 340 nm.The function of time was monitored in a Cary 1/3 UV/VIS dual beam spectrophotometer.

Statistical analysis: Data are expressed as Mean±SEM. Differences were tested for statistical significance using two-way Analysis of Variance (ANOVA). Individual differences between groups were assessed using the Tukey's Studentized Range test (SAS Institute, Cary, NC). Significant differences in means were accepted at $p = 0.05$.

RESULTS

Modifications were made in the diets administered (which included levels of sucrose, dextrose and alphacel) to ensure that all diets were isocaloric. There were no differences in feed intake among the different groups. The average weight gains were 239, 242 and 238 g for the C, C+50% CGJ and C+10% raisins. In the groups fed C, C+CGJ, C+Raisins, there was a small increase in cecal weight from 1.2, 1.6 and 1.8 and there were no differences in pH with means of 8.10±0.04 for C+50% CGJ; 8.02±0.05 for C+10% raisins and 7.90±0.06 for control.

ACF enumeration: There were significant ($p<0.05$) differences in ACF between rats fed control and treatment diets. ACF were higher in the distal colon ($p<0.05$) than in the proximal colon (Fig. 1). The number of ACF were significantly lower in treatment groups compared to controls with means ranging from 27.58±1.71 for rats fed C+50% CGJ, 34.93±2.13 for rats fed C+10% raisins and 102.80±9.01 for rats fed control, respectively (Fig. 1). The total number of crypts in treatment rats were significantly different from the control with 93.62±4.3 for rats fed C+50% CGJ, 116.66±5.3 for rats fed C+10% raisins and, 358±13.3 for control (Fig. 2) The number of ACF containing 3, 4 and >5 crypts were significantly ($p<0.05$) higher than ACF with 1 and 2 crypts (Fig. 3). The percent reductions in ACF compared to the control were 73 and 66% for diets containing C+50% CGJ and C+10% raisins, respectively (Fig. 4).

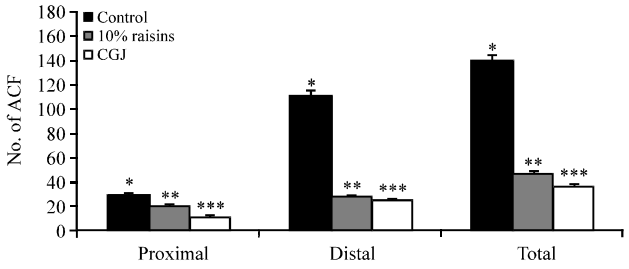

Fig. 1: Effect of grape products on colonic Aberrant Crypt Foci (ACF) in Fisher 344 male rats, The data displayed with Mean±SEM (bars). Significant differences were at $p<0.05$, respectively. (CGJ) concord grape juice, *Mean±SEM are significantly different, **Denotes a significant difference from control, ***Denotes a significant difference from control

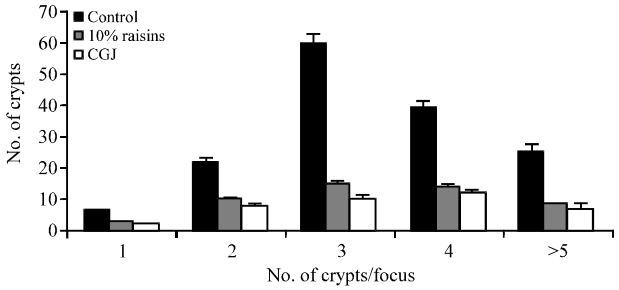

Fig. 2: Effects of grape products on total colonic aberrant crypts in Fisher 344 male rats. The data are displayed with Mean±SEM (bars). Significantly different were at $p<0.05$, respectively. (CGJ) concord grape juice

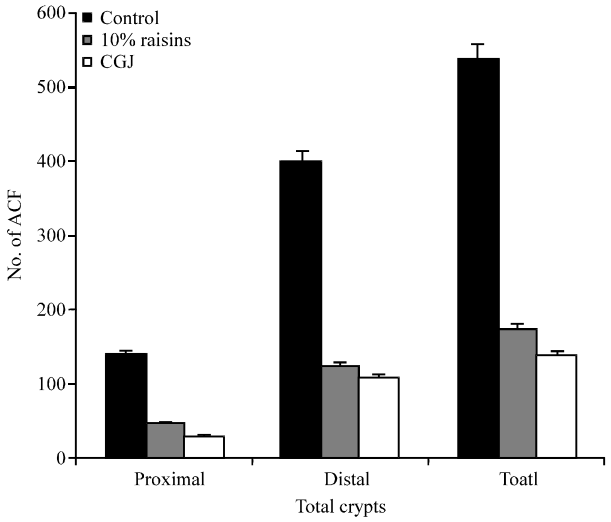

Fig. 3: Effects of grape products on number of crypts per focus in Fisher 344 male rats. The data are displayed with Mean±SEM (bars). Significant differences were at $p<0.05$, respectively. (CGJ) concord grape juice

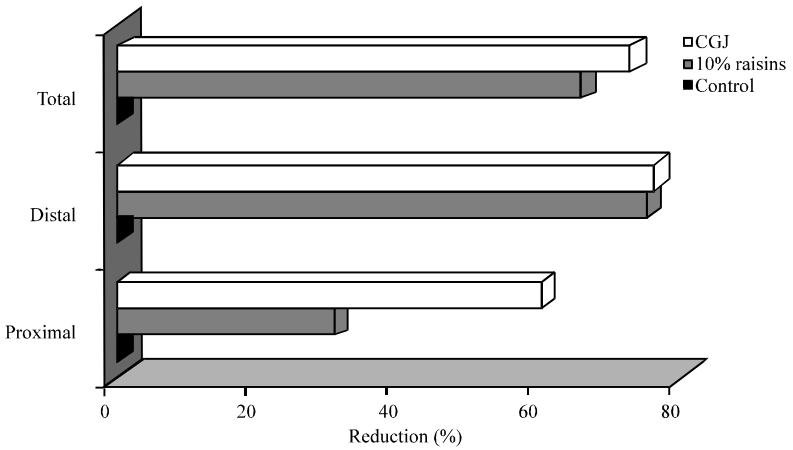

Fig. 4: Percentage reductions of grape products in aberrant crypt foci compared to control. Total percentage reduction for groups consuming C+50% CGJ and C+10% raisins is 73.2 and 66.01, respectively. (CGJ) concord grape juice

Glutathione S-transferase: The compounds detoxified by GST's are diverse and include a number of chemical carcinogens, anticancer drugs and environmental chemicals. There are many varieties of GST inducers including the polyphenols in grapes. There was a significant ($p<0.05$) increase in total activity among the groups. The most significant difference was seen in the groups fed C+50% CGJ as compared to the control. The total activity of Glutathione S-transferase (GST) was 7.55±0.7, 22.66±1.88 and 16.38±2.02 for the groups consuming diets containing C, C+50% CGJ and C+10% raisins, respectively.

The diets in this study were modified to be isocaloric (Table 1).

DISCUSSION

In a review article, Greenwald *et al.* (2001) reported that evidence for dietary recommendations that involve vegetable and fruit intakes remains strong, however the data supporting other foods remain less convincing. For fruits and vegetables, the majority of case-control studies indicate the support for reduced risk of cancer when consumption is consistent. Therefore, demonstrating that fruits and vegetables which are rich in antioxidants and other micronutrients and have a protective effect against diverse cancers, including lung, esophageal, oral, laryngeal, cervical and breast. There are a variety of methods in which these micronutrients may exert their ability to block DNA damage, mutation and carcinogenesis by oxygen radicals, Poly Aromatic Hydrocarbons (PAHs) and other chemical carcinogens (Perera, 1997). The ACF were enumerated using the procedure by Bird (1987). The number of ACF were higher in the distal colon ($p<0.05$) than in the proximal colon. The data reported is consistent with other reports that the distal colon shows a greater incidence of colorectal cancer than proximal colon in humans. Challa *et al.* (1997) also reported that there was a significantly higher number of ACF in the distal portion of the colon than in the proximal. There were significant ($p<0.05$) reductions in total number of colonic ACF, as well as number of crypts (Fig. 2). Compared to the controls, C+50% CGJ and C+10% raisins had a significant ($p<0.05$) effect in reducing ACF but C+50% CGJ had a greater effect on ACF which may be due to the increased bioavailability of polyphenolic compounds. Challa *et al.* (1997) reported that green tea showed a significant ($p<0.05$) reduction in the number of ACF. This clearly emphasizes that foods containing polyphenols can have a great impact on deterring the growth of preneoplastic lesions. There was a greater reduction in 3, 4 and 5 crypts/foci, which is significant, because studies conducted using end-point tumor models suggest that ACF containing 3, 4 and 5 crypts/foci sustain and become tumors in long-term studies (Fig. 3). The percent reduction of ACF in the group consuming C+CGJ (50%) was 7% higher than the group consuming C+raisins (10%). Feeding raisins at 10% did not affect cecal pH but slightly increased cecal weight, which maybe a result of the production of short-chain fatty acid from fermentation of fiber. Verghese *et al.* (2002) discussed that the elevation in cecal weight due to ingestion of inulin or fructooligosaccharides diets may be a direct result of the short-chain fatty acids promoting cecal growth. In addition, Ferguson *et al.* (2004) discussed that the inhibition of cell proliferation in selected breast cancer cell lines were due to flavonoid extractions in concentrations ranging from Mertens-Talcott *et al.* (2003) reported that quercetin as well as ellagic acid reduced cell proliferation but had a greater synergistic effect on the cells when combined. Therefore, phytochemicals have a greater synergistic interaction against cancer cell proliferation when consumed together. Results of this study have shown that feeding CGJ and raisins did play a large role in reducing AOM-induced ACF in Fisher 344 male rats. Specifically, there was a greater percentage reduction in the distal colon than in the proximal (Fig. 4). This observation is significant because most colon tumors develop in the distal portion of the colon rather than the proximal. Glutathione S-transferases (GSTs) are a group of multigene enzymes that are essential and encompass approximately 2-4% of cytosolic proteins (Eaton and Bammler, 1999). Hayes and Pulford (1995) demostrated that many compounds detoxified by GST's are mutagenic and the level of GST expression can be a key determinant of sensitivity to carcinogenesis. Challa *et al.* (1997) stated that green tea did not affect the activity of GST, however in this study feeding CGJ (50%) and raisins (10%) did significantly ($p<0.05$) increase the level of GST activity. The results from this study also indicate that GST levels for rats fed C+50% CGJ and C+10% raisins were significantly ($p<0.05$) higher than the control and was also

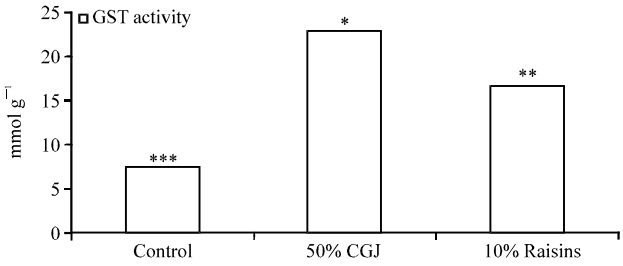

Fig. 5: Levels of Glutathione S-transferase activity in AOM treated rats fed control, C+50% CGJ and C+10% raisins induced by the effects of grape products. Values are Mean±SEM, differences were significant at $p<0.05$ (CGJ) Concord grape juice

significantly different from each other (Fig. 5). GST total activity increased by C+CGJ (50%) was six percent higher than the GST activity level for rats fed C+raisins (10%). The results suggest that CGJ and raisins may have catalyzed the detoxification of DNA binding electrophiles by which the induction of ACF was reduced.

CONCLUSION

The results of this study demonstrated that feeding grape products in the form juice (50%) and raisins (10%) significantly reduced the azoxymethane induced aberrant crypt foci in Fisher 344 male rats. The protective effects of grapes against chemically induced colon cancer might be through induction of Glutathione-S-tranferase enzyme. Long term feeding of grape products in end point tumor model can be used in future to understand the mechanisms and their protective effects on progression stage of colon cancer.

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