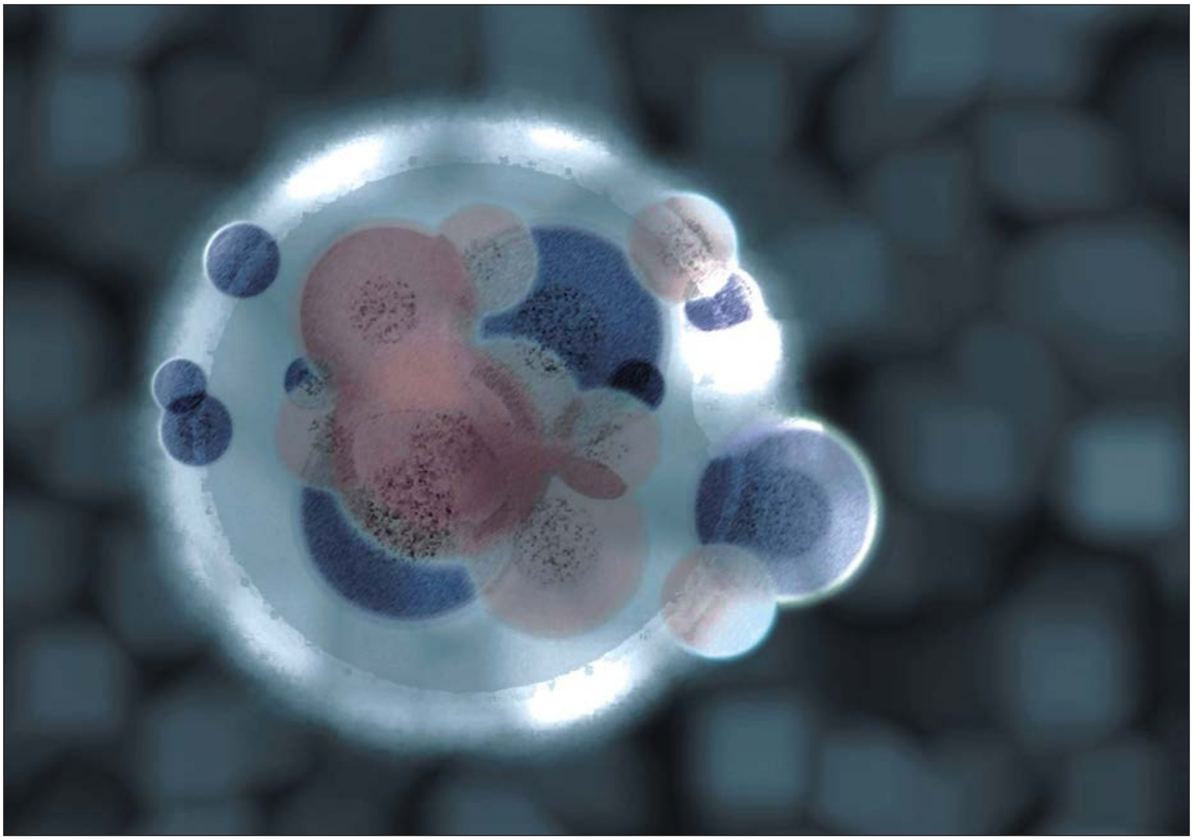

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Canola and Mustard Seeds/Sprouts Reduce the Formation of Aberrant Crypt Foci in Azoxymethane-Induced Colon Cancer in Fisher 344 Male Rats

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Abstract: The aim of the current study was to determine the efficacy of selected canola or mustard seeds (S) and canola sprouts (SP) cultivars (5 and 10% levels) on azoxymethane (AOM) induced Aberrant Crypt Foci (ACF) in Fisher 344 male rats. Following a one week period of acclimatization Fisher 344 male rats were randomly assigned to groups (n = 6). At seven and eight weeks of age rats received two successive s/c injections of AOM in saline at 16 mg kg^{-1} body weight. Rats were killed by CO_2 asphyxiation at 17 weeks of age. The number of ACF in the proximal and distal colon in the treatment groups ranged from 6.33 (5% Pacific gold) to 15.55 (5% Jetton) and 21.33 (10% Pacific gold) to 52.0 (5% Idagold). Glutathione-S-transferase activity (µmol mg^{-1}) was significantly ($p<0.05$) higher in the groups fed canola or mustard seeds compared to the control. Among the treatment groups the highest GST activity (µmol mg^{-1}) was observed in the rats fed 10% Pacific gold group (32.50). The results from this study show that canola or mustard sprouts and seeds reduced the incidence of AOM-induced ACF and may possibly prevent the incidence of colon cancer.

Key words: Omega-3 PUFA, aberrant crypt foci, azoxymethane, canola

INTRODUCTION

Cancer is the second leading cause of death in the United States following heart disease. The American Cancer Society (ACS) estimates over 560,000 deaths annually due to cancer (ACS, 2007). Colorectal cancer is cancer initially localized in the colon and rectum and is the third most common cancer found in men and women in the US. The ACS estimates that there will be approximately 106,680 new cases of colon cancer and 41,930 new cases of rectal cancer in 2007 in the U.S. The incidence of colon cancer shows an annual increase of 3-7% especially in developed countries where intakes of the Western diet are prominent.

Fruits and vegetables have been shown to have a strong protective effect against certain types of cancers such as colon cancer (Temple and Gladwin, 2003). It is estimated that a daily consumption of five servings of fruits and/or vegetables could lead to a 20% decrease in cancer rates (Keck and Finley, 2004). Vegetables belonging to the cruciferous family such as cabbage, broccoli and brussel sprouts contain phytochemicals and other components that help prevent the development of cancer (Barrett *et al*., 1998).

Canola (rapeseed), a member of the cruciferous family and *Brassica* sp., is a specialty crop grown predominately in Canada but is now promoted in many areas of the US as a major cash crop. Canola sprouts and seeds (*Brassica napus* and *Brassica campestris*) are rich sources of omega-3 (n-3) PUFA and other phytochemicals such as isothiocyanates (ITCs) and indoles. Both *in vivo* and *in vitro* studies have shown that n-3 fatty acids present in fatty fish and fish oils may inhibit carcinogenesis (Larsson *et al*., 2004). Cruciferous vegetables contain a variety of different glucosinolates and each forms a different isothiocyanate when hydrolyzed. The conversion or breakdown of glucosinolates to isothiocyanates occurs by the enzyme myrosinase as well as human intestinal microorgamisms. Mustard seeds (*Sinapis alba* and *Sinapis juncea*) are

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commonly used as spices or as an alternative medicine in some cultures. The bitter, pungent odor and taste that is characteristic to mustard is due to the presence of phytochemicals such as isothiocyanates (Velisek *et al*., 1995). These compounds have been shown to have anti-neoplastic activity thus possibly providing benefits in chemoprevention (Eskin *et al*., 2007).

Aberrant Crypt Foci (ACF) are lesions found in the colon that are considered precursors to the development of colon cancer. ACF in human colons have similar morphological and genotypic features as those seen in animal colons and tumors (Adler *et al*., 2002). Azoxymethane (AOM) is a widely used rodent colon-specific carcinogen used to induce the development of ACF in the Fisher 344 rat model used for this experiment. In addition to ACF, the number of crypts/focus can also be used as a screening tool. Glutathione-S Transferase (GST) is a phase II detoxifying enzyme which can modulate the risk of cancer by formation of polar (water soluble) conjugates of carcinogens that are easily excreted in the urine or bile, thus reducing the load of the carcinogen. High GST activity in the liver may be responsible for a reduction in the risk of cancer formation (Hayes and Strange, 1995).

The reports on chemopreventive potential of canola and mustard seeds/sprouts are scarce, therefore the objective of this study was to investigate the potential of canola on azoxymethane induced ACF formation and GST activity, a crucial phase II detoxification enzyme, in Fisher 344 male rats.

MATERIALS AND METHODS

Animals and housing: Fisher 344 male weanling rats (Harlan, IN) were housed in stainless steel wire cages at two rats per cage. Following a one week acclimatization period, rats were randomly assigned to groups. Thirteen groups of six rats were assigned to selected dietary treatments based on the AIN-93 diet (Reeves *et al*., 1993a, b) the control group (C) received a basal AIN-93G diet and the rats in the treatment groups received diets consisting of C + 5 and 10% of selected cultivars of canola/mustard seeds or sprouts). Seeds and their respective sprouts were studied to determine whether sprouting would have any beneficial effect in enhancing the chemopreventive potential of the canola and mustard seeds. Jetton (*Brassica napus*) was the canola cultivar used. The mustard cultivars were Ida gold (*Sinapis alba*) and Pacific gold (*Sinapis juncea*). The temperature and relative humidity were maintained at 21°C and 50%, respectively. Light and dark cycles were 12 h each and feed and water were provided *ad libitum*. At 13 weeks, rats were killed using CO_2 asphyxiation. Daily feed consumption and bi-weekly body weights were recorded. All protocols involving animals were approved by the Institutional Animal Care and Use Committee, Alabama A and M University.

Chemicals: All chemicals except azoxymethane (Midwestern Research Institute, NCI Repository, Kansas City, MO) were obtained from Sigma Chemical (St. Louis, MO).

Carcinogen injection: For induction of Aberrant Crypt Foci (ACF), all animals received two subcutaneous injections of azoxymethane (AOM) in saline at 16 mg kg^{-1} body weight at seven and eight weeks of age.

Colon sample collection and aberrant crypt foci (acf) enumeration: At 17 weeks of age, rats were killed using CO_2 asphyxiation after an overnight fast. Colons of rats were removed and livers were collected. Liver samples were stored at -80°C until further analysis. Potassium phosphate buffer (0.1 M, pH 7.2) was used to flush the colons and ACF was enumerated as described by Bird (1987).

Glutathione S-Transferase (GST) assay: The protocol described by Habig *et al.* (1974) was followed. Assay sample (50-100 μL) was analyzed using Cary1/3 UV/VIS dual beam spectrophotometer at 340 nm.

Statistical analysis: Data are expressed as means ± SEM and were analyzed using the SAS statistical program by analysis of variance (ANOVA) and means were separated using Tukey's studentized range test. Differences were determined for statistical significance using two-way ANOVA. Differences were considered significant at $p<0.05$. Correlation between GST activity and ACF was analyzed by Pearson correlation using SPSS.

RESULTS AND DISCUSSION

Weight gain and daily feed intake: Weight gain was significantly ($p<0.05$) lower in the control group compared to the rats fed the treatment diets, however, there were no significant differences in feed intake among rats fed control and the rats fed canola/mustard diets (Table 1). No significant differences were seen in weight gain or feed intake among rats fed canola/mustard diets (Table 1).

Aberrant Crypt Foci (ACF): ACF incidence in the proximal and distal colon in rats fed the treatment diets (i.e., Jetton, Ida and Pacific gold seeds and sprouts) were

Table 1: Effect of canola and mustard seeds/sprouts on weight gain and daily feed intake in Fisher 344 male rats

Groups	Weight gain (g/13 weeks)	Feed intake (g day^{-1})
Control	148.33±23.02[b]	12.73±0.26[a]
5% JS	178.67±9.02[a]	13.15±0.63[a]
10% JS	155.83±26.68[a]	13.17±0.89[a]
5% IS	173.0±3.46[a]	12.21±0.31[a]
10% IS	165.33±10.83[a]	11.18±0.92[a]
5% PS	153.00±3.61[a]	13.08±0.67[a]
10% PS	156.50±18.11[a]	12.46±0.72[a]
5% JSP	166.33±5.55[a]	13.33±0.57[a]
10% JSP	162.83±6.34[a]	13.22±1.65[a]
5% ISP	171.33±4.33[a]	13.15±0.63[a]
10% ISP	179.17±5.22[a]	13.01±2.04[a]
5% PSP	170.36±4.33[a]	13.29±1.41[a]
10% PSP	171.17±5.31[a]	12.91±1.63[a]

S: Seed, SP: Sprouts, J: Jetton, I: Idagold, P: Pacificgold, Values are means±SEM, [ab]: Means in a column with the same superscript do not significantly differ (p<0.05)

Table 2: Aberrant Crypt Foci in Fisher 344 male rats fed canola/mustard seeds and sprouts

Groups	Proximal colon	Distal colon	Total colon
Control	41	92	133
5% JS	14	30	34
10% JS	16	26	42
5% IS	9	30	39
10% IS	7	28	35
5% PS	11	28	39
10% PS	13	25	39
5% JSP	15	30	45
10% JSP	16	24	40
5% ISP	8	52	60
10% ISP	11	44	55
5% PSP	6	28	34
10% PSP	11	21	32

Abbreviations-S-seed, SP: Sprouts, J: Jetton, I: Idagold, P: Pacificgold, [ab]: Means in a column with the same superscript do not significantly differ (p<0.05)

significantly (p<0.05) lower compared to the control group (Table 2). In all the groups, ACF was significantly (p<0.05) higher in the distal colon compared to the proximal colon. This is also seen in humans where ACF incidence is higher in the distal compared to the proximal colon. These data are also consistent with findings of Bird (1987) that the distal colon shows a greater incidence of colorectal cancer than the proximal colon. While there were no significant differences in the number of proximal and distal ACF in rats fed the treatment diets, ACF incidence ranged from low of 6 in the proximal colon of rats fed 5% Pacific gold sprouts to a high of 16 in the groups fed 10% Jetton sprouts and seeds. However, in the distal colon, the highest number of ACF were seen in the group fed Ida gold cultivar (sprouts and seeds). The lowest incidence of ACF was observed in the group fed Pacific gold sprouts, compared to the control, rats in this group had a 74 and 76% decrease for rats fed 5 and 10% levels, respectively.

Total aberrant crypts: Total aberrant crypts in rats fed canola/mustard seeds and sprouts were significantly (p<0.05) lower than the control group. The greatest reductions were seen in the rats fed the Pacific gold cultivar at 5 and 10% levels which resulted in 76% and 77% decreases compared to the control group (Fig. 1). Total aberrant crypts take into account the number of ACF as well the crypts per focus and therefore give a clear picture of chemopreventive effects of foods.

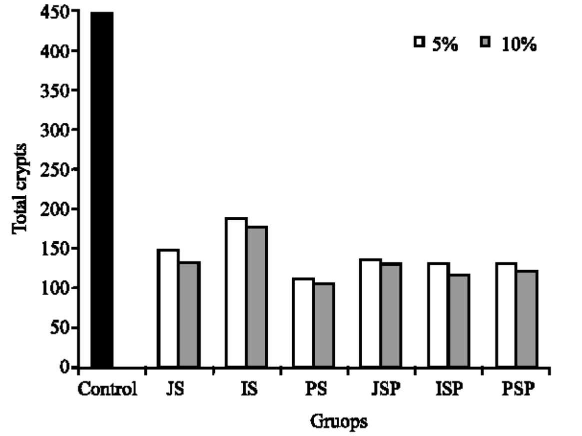

Fig. 1: Total aberrant crypts in colon of fisher 344 male rats fed canola/mustard seeds and sprouts

Table 3: Glutathione-S-Transferase (GST) activity in liver of rats fed canola/mustard seeds and sprouts

Groups	GST activity (μmol mg^{-1})
Control	18.31±1.2[c]
5% JS	32.60±4.9[a]
10% JS	34.76±3.2[a]
5% IS	31.91±3.4[a]
10% IS	34.30±2.9[a]
5% PS	35.89±3.3[a]
10% PS	37.60±3.7[a]
5% JSP	21.15±0.2[b]
10% JSP	24.50±1.9[b]
5% ISP	23.08±1.4[b]
10% ISP	24.70±2.0[b]
5% PSP	30.19±2.7[a]
10% PSP	32.50±3.9[a]

Abbreviations-S-seed, SP: Sprouts, J: Jetton, I: Idagold, P: Pacificgold, Values are means±SEM, [abc]: Means in a column with the same superscript do not significantly differ (p<0.05)

Glutathione s-transferase activity: GST activity was significantly (p<0.05) higher in rats fed canola and mustard seeds compared to the control group (18.31). The highest GST activity was seen in rats fed 10% Pacific gold seeds (37.60) among those fed canola and mustard containing diets (Table 3). Feeding canola and mustard sprouts resulted in significantly (p<0.05) higher GST activity when compared to the control group (18.31). Rats fed 10% Pacific gold sprouts diet had the highest GST activity (32.50) among all sprout treatment groups (Table 3). GST activity in the groups fed treatment diets ranged from a low of 21.95 in rats fed 5% JSP to a high of 37.60 in rats fed 10% PS.

The aim for the current study was to determine the effects of feeding canola sprouts and seeds at selected concentrations (5 and 10%) on AOM induced ACF in F344 male rats. ACF, which are putative preneoplastic lesions, are used as a biomarker in short-term carcinogenesis and chemoprevention studies. Results from our data suggest that compared to the control group, rats given canola sprouts and seeds had significantly ($p<0.05$) lower incidence of ACF and total aberrant crypts; however, the precise mechanism of preneoplastic lesion inhibition is not fully understood. As this is the first study to our knowledge, to investigate the chemopreventive potential of canola sprouts and seeds on an animal model, we can assume that the protective effects offered by canola sprouts and seeds maybe attributed to its phytochemical content.

As a member of the cruciferous family and *Brassica* sp., canola is a rich source of phytochemicals. The seeds in particular are important sources of omega-3 (n-3) PUFA specifically alpha-linolenic acid (ALA). Omega-3 (n-3) PUFA are capable of decreasing the production of free radicals and reactive oxygen species as well as suppress arachidonic acid (AA, 20:4n-6) which may result in altered immune response to cancer cells and modulation of inflammation, cell proliferation, apoptosis, metastasis and angiogenesis (Larsson *et al*., 2004). Other observations include the inhibition of eicosanoid biosynthesis by arachidonic acid (AA), an omega-6 fatty acid derived from linolenic acid (LA) by the action of cyclooxygenase-2 (COX 2) (Rose and Connolly, 1999). A study conducted in our laboratory showed that rats fed flax seed meal and flax seed oil which are rich sources of omega-3 (n-3) PUFA significantly ($p<0.05$) suppressed the incidence of AOM-induced ACF (Williams *et al*., 2007). Similarly, Coleman *et al*. (2002) reported that rats fed 20% fish oil had 19% fewer ACF ($p<0.05$) in their colon with reductions of 24% reduction ($p<0.05$) in the proximal colon and 15% reduction ($p<0.064$) in the distal colon.

Among the prominent constituents contained in Canola sprouts and seeds is both soluble and insoluble fiber. Insoluble fiber helps improve laxation mainly by increasing fecal bulk and reducing bowel transit time and hence reduce mucosal exposure to potential carcinogens or tumor promoters. Insoluble fibers, relative to soluble fibers, largely resist fermentation and have been shown to be more protective against colon cancer in animal models (Cameron *et al*., 1989; McIntosh *et al*., 1996) and in humans although the evidence relating to these findings still remains inconsistent (Kim, 2000).

Isothiocyanates (ITC) which are present in canola and mustard seeds and sprouts have shown a protective role in the prevention of various cancers (Zhang, 2005). Cruciferous vegetables which contain ITCs have been shown to increase the 2OHE1:16aOHE1 ratio thus reducing the growth of cancer cells (Keck and Finley, 2004). In colorectal cancer, ITCs may be beneficial by aiding in the elimination of carcinogens from the body.

In this study, we saw a significant ($p<0.05$) increase in phase II GST activities in rats fed canola sprouts and seeds compared to the control. ITCs role in the prevention of disease may also include their ability to inhibit cytochrome enzymes in phase I biotransformation in addition to being inducers of phase II enzymes such as Glutathione-S-Transferase (GST) (Bonnesen *et al*., 2001) which was seen in this study. In the current study we found a strong correlation between the incidence of ACF and GST activity in the seeds ($R^2 = 0.927$, $p<0.05$) and sprouts ($R^2 = 0.720$, $p<0.05$) (Table 4). The relationship between ACF and GST activity in Jetton, Ida and Pacific gold cultivars are shown in Fig. 2. In the rats fed Jetton,

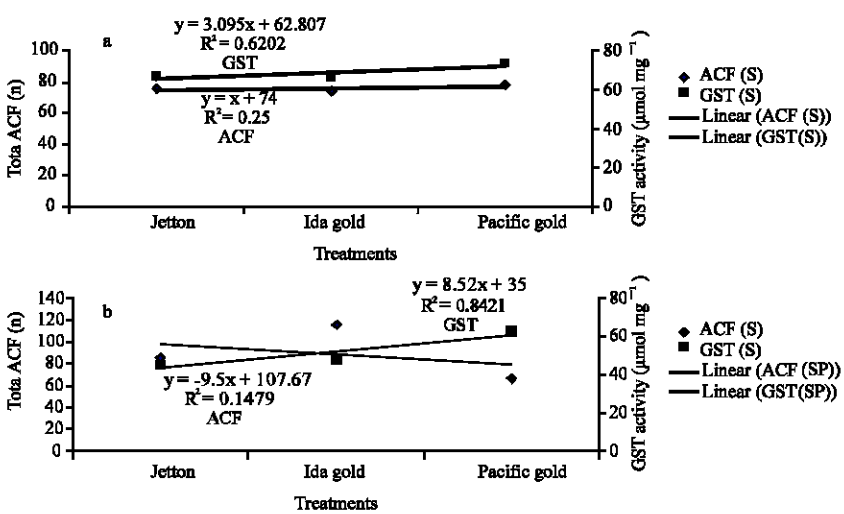

Fig. 2: Correlation between total ACF (y-axis) and GST activities (z-axis) in rats fed canola/mustard seed (a) and sprouts (b)

Table 4: Correlation relationship between total ACF and GST activity in rats fed canola/mustard seeds and sprouts using Pearson correlation coefficient

	Total ACF* (n = 3)	GST (μmol mg^{-1})* (n = 3)	R^2
Canola seeds (JIP)	76.00	68.9	$R^2 = 0.927$
Canola sprouts (JIP)	88.67	52.04	$R^2 = 0.720$

*: Average values for black-eyed peas, kidney beans, pinto beans and soybeans were used

Ida and Pacific gold seeds, GST activity was lower (Fig. 2a) compared to the groups fed Jetton, Ida and Pacific gold sprouts (Fig. 2b). This suggests that with an increase in GST activity the number of total ACF was lower in Jetton, Ida and Pacific gold sprouts.

In present study, data showed that canola and mustard sprouts/seeds significantly ($p<0.05$) reduced the incidence of AOM induced ACF and therefore may possibly have potential in the prevention of colon cancer. There is a need to further define the molecular mechanisms of ITCs at the cellular level, especially the role of glucosinolates. The feasibility of consumption and use of ITCs for chemoprevention in individuals that are at high risk of cancers should be further investigated. These findings could possibly lead to the recommendation of consumption of canola in various foods such as salads, snack bars, muffins, or other baked foods which could possibly play a significant role in dietary chemoprevention. As underexploited crops in the US, this data helps to focus and emphasize the benefits of canola and mustard and their importance in health and possible role in prevention of colon cancer.

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