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Preventive Effects of Oat Bran Extracts on Rat Colon Carcinogenesis Induced by 1,2-Dimethylhydrazine

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Abstract The effect of oat bran extracts on the formation of aberrant crypt foci (ACF) in the colon induced by 1,2-dimethylhydrazine (DMH) was studied in F344 male rats. Extracts were prepared using various combinations of temperature (40, 45, 50, 55, or 60°C: X_1), ethanol concentration (0, 5, 10, 15, or 20%: X_2), and pH (5, 6, 7, 8, or 9: X_3). Among the various extracts tested, one ethanol extract (EE; 45°C, 15% ethanol at pH 6) and one water extract (WE; 50°C at pH 5) were selected based on their *in vitro* antitumor activity. The animals were fed with basal diet alone or basal diet supplemented with 0.25 or 0.5% of EE or WE for 6 weeks. During the initial 2 weeks of the 6-week test period, the rats were subcutaneously injected with DMH (30 mg/kg) 4 times for the induction of ACF. DMH induced an average of 322.7 and 142.9 aberrant crypts (AC) and ACF, respectively. A low dose (0.25%) of EE (containing 38.3% β-glucan) and WE (containing 22.8% β-glucan) greatly reduced the numbers of DMH-induced AC and ACF. Significantly, ACF consisting of more than 3 AC were reduced by half in which the effect of EE, containing a higher concentration of β-glucan, was superior to that of WE. These results demonstrate that oat bran extracts may confer protection against colon carcinogenesis.

Keywords: oat bran extract, β-glucan, aberrant crypt foci (ACF), colon carcinogenesis

Introduction

Oats have long been known as a cereal grain with physiological benefits, including the lowering of blood cholesterol and glucose, and cancer prevention, especially with respect to colon cancer. Oat β-glucan, the main component of oat soluble fiber, is believed to play important roles in these physiological effects (1, 2). Oat β-glucan is a linear non-starch polysaccharide consisting of (1 → 3)(1 → 4)-β-D-linked glucose units (3), and is located in endosperm cell walls, which are thickest in the subaleurone layer adjacent to the aleurone layer. Oat bran consists of the outer layers of the groat, which contain the majority of dietary fiber, and is prepared by separating the outer layers of groats from the starch-containing endosperm by mechanical milling and separation. When the bran is separated, β-glucan is enriched in the bran fraction (4).

Similar to other dietary fibers, possible mechanisms by which β-glucan may inhibit colon carcinogenesis include regulating the speed of colonic transit and the consistency and weight of stool, thereby diluting carcinogens and altering microbial metabolism (1). Since mammalian enzymes are unable to hydrolyse β-glucan, it remains nearly intact in the small intestine and is partially degraded in the human large bowel, especially the cecum, by bacteria. In the colon, β-glucan produces structural and functional changes in the intestinal epithelium and modifies rates of colonic cell proliferation and migration. Microbial fermentation of β-glucan within the large bowel results in the production of volatile fatty acids such as acetate, propionate, and butyrate, and a lower luminal pH, which in turn affects colonic microbial populations and their metabolic activities (2, 5).

The degradation and metabolism of β-glucan in the intestinal tract may be influenced by the physicochemical and structural characteristics of β-glucan, such as its viscosity, solubility, molecular weight, and the ratio of β-(1 →3):β-(1 →4) bonds (1-5). Physiological effects may very depending on β-glucan metabolism in intestinal tract. Highly viscous β-glucan enhances its effect on plasma glucose and insulin levels following an oral glucose load (6). Different fibers have different effects on mucosal growth and cell proliferation in the small intestine (7). High β-glucan solubility is associated with the enhancement of fermentation in the cecum by colonic bacteria, producing short fatty acids which are reabsorbed in colon and used as metabolic resources (5). The characteristics of β-glucan are dependent on the extraction conditions, and the methods of precipitation and purification (8-11).

Although a high fiber diet correlates with a lower frequency of large bowel cancer, epidemiological studies have been unable to find evidence to support a hypothesis or mechanism by which fiber provides protection from colon cancer. Several studies have also reported a lack of consistency regarding the effects of various fiber sources on colon carcinogenesis with reports of inhibition, no effect, and even tumor enhancement (7, 12-14). One study

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reported that the modulation of small intestine mucosal structure and growth due to fiber appeared to be affected by alterations in cell proliferation, and to depend not only on the quantity but also the quality of the fiber (7). Another study, in contrast, reported that there was no evidence that the overall chemical composition and structure of various fibers are related to the gastroenterological effects they produce (13). Moreover, the consumption of oat bran was associated with the enhancement of proximal colon carcinogenesis despite the acidification of colon content (14). However, studies on the anticancer effects of oat β-glucan or extract, not oat bran, have not been reported.

The present study was therefore done to investigate the preventive effects of oat bran extracts on rat colon carcinogenesis induced by 1,2-dimethylhydrazine (DMH). This was done by the quantitative assessment of preneoplastic aberrant crypt foci (ACF) in the colonic mucosa, and to determine the optimal extraction conditions to produce oat bran extracts providing the highest level of protection from colon cancer.

Materials and Methods

Preparation of oat bran extract Extracts from oat bran were prepared by the procedure described by Jeong *et al.* (15). Extraction was carried out with various combinations of extraction temperature (40-60°C), solvent concentration (0-20% ethanol) and solvent pH (5-9). Based on preliminary *in vitro* antitumor activity (16), the following two extracts were selected for further investigation. Ethanol extract (EE) was produced with 15% ethanol at a temperature of 45°C, and pH 6, and water extract (WE) was produced with distilled water at 50°C and pH 7.

Anlaysis of physicochemical composition The starch and crude protein content were analyzed by the AOAC procedure (17). The β-glucan content of each oat bran extract was measured using a β-glucan assay kit (Megazyme Pty, Ltd., Ireland). The apparent viscosity of aqueous solutions of oat bran extracts was measured with a viscometer (Brookfield DV-II+; Brookfield engineering laboratories, Inc., MA, USA) at a shear rate of 39.6/sec at 4°C. Extracts (0.2 g/20 mL) were dissolved in distilled water by stirring at 55-60°C for 2-3 hr. After solubilization, undissolved materials were removed by centrifugation (2,000×g, 10 min) (18). Apparent viscosity of the supernatant was measurd.

High-performance size-exclusion chromatography (HPSEC) Average β-glucan molecular weight in each oat bran extract was estimated by using HPSEC. The HPSEC system included an Ultrahydrogel™ mixed bed linear column (7.8×300 mm; Waters, Milford, MA, USA) maintained at 35°C and a RI detector (Waters 410; Waters). Sample (1.5 g/mL) was dissolved in sodium nitrate (0.1 M) by heating (60°C) and stirring (120 rpm, 15 hr), and filtered through a syringe filter (0.2 μm; Millipore Co., Bedford, MA, USA).Twenty μL of dextran standard (Sigma Chemical Co., St. Louis, MO, USA) or extract sample was injected and eluted at 0.7 mL/min with sodium nitrate (0.1 M) (11).

Determination of the β-(1→3) : β-(1→4) bond ratio Analysis of the β-(1→3):β-(1→4) bond ratio was carried out by a modification of the method of Izydorczyk *et al.* (11). Oat bran extracts (2 mg/mL) were dissolved in sodium phosphate buffer and then mixed with lichenase (4 U; (1→3), (1→4)-β-glucan-4-glucanohydrolase, Megazyme Pty, Ltd.). The mixture was incubated at 40°C for 22 hr followed by mixing with 5 mL of sodium acetate buffer, and centrifugation at 2,000×g for 10 min. A 0.1 mL aliquot of supernatant was mixed with exo-(1→3)-glucanase (1 U; Sigma Chemical Co.) and incubated at 40°C for 1 hr. Free glucose content released from β-(1→3) bond breakdown was analyzed by measuring the absorbance at 510 nm after incubation with glucose oxidase/peroxidase/4-amino-antipyrin (GOPOD; Megazyme) reagent at 40°C for 20 min. Total glucose content in β-glucan was determined by using the β-glucan assay kit. The amount of glucose released from the breakdown of the β-(1→4) bond in β-glucan was estimated by subtracting the glucose released from β-(1→3) bond break-down from the total glucose content.

Animals and diets The animals used for this study were 5-week-old male F344 rats (SLC Co., Shizuoka, Japan). They were housed in a controlled animal facility room with a 12:12-hr light/dark cycle, a temperature of 23±2°C, and a humidity level of 55±10%. Rats were allowed to feed freely on the basal diet (CRF-1; Charles River Japan, Yokohama, Japan) and water. After acclimatization for 2 weeks, F344 male rats showing stable body weight gain and no clinical symptoms were randomly divided into seven groups by the body weight. The average of body weight among the groups was uniform.

Experimental treatment Experimental animals were divided into 4 groups fed various oat bran extracts at doses and 3 control groups shown in Fig. 1. The animals were fed basal diet alone or supplemented with 0.25 or 0.5% EE or WE for 6 weeks. During the 2 weeks before the 6-week test period, all rats, except for rats in 2 negative control groups, were subcutaneously injected with DMH (30 mg/kg) a total of 4 times. Negative control rats were fed 0.5% EE or WE without DMH injection, and positive control rats were injected with DMH while fed a basal diet. Body weights and food intake were recorded once per week.

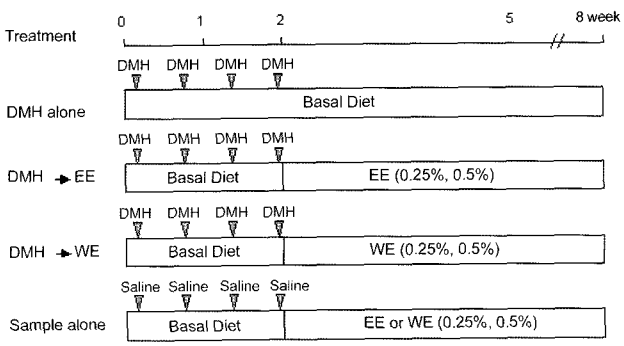

Fig. 1. Protocol of colon carcinogenesis induction to test the preventive effects of oat bran extract. Samples produced with different extraction conditions: EE, ethanol extract; WE, water extract.

After 8 weeks including 2 weeks of aberrant crypts (AC)-inducing periods and 6 weeks of test periods, all animals were sacrificed and their liver, spleen, and kidneys were weighed. In order to examine preventive effects regarding colon cancer, the large intestine was removed, and AC and ACF on the inner surface of the colon were inspected.

Analysis of colonic AC and ACF AC and ACF in the colon were measured by the procedure of Bird (19). For counting AC and ACF, colons were removed, gently inflated by pouring a 1:1 mixture of 0.9% sodium chloride (Sigma) solution and 10% formalin (Sigma) into the colon, and fixing with 10% neutral phosphate-buffered formalin for 10 min. The colons were then cut longitudinally from anus to cecum, and spread flat on a filter paper for further fixation. After staining with 0.2-0.5% methylene blue solution for 30 sec, AC and ACF per rat were counted under a light microscope.

Histological examination The major organs, including the liver, spleen and kidney were fixed in 10% neutral phosphate buffered formalin, and embedded in paraffin for routine processing and examination of H&E-stained sections.

Statistical analysis The *in vivo* test results were expressed as means ± standard deviation (SD). The significant differences between group means were assessed using the Student's *t*-test (20).

Results and Discussion

Physicochemical characteristics of oat bran extracts A series of oat bran extracts produced under various conditions of temperature, solvent and pH were tested for anti-tumor activity *in vitro* (16). Based on these preliminary results (data not shown), 2 extracts were chosen for further investigation regarding their protective effects against colon cancer *in vivo*: extract produced with 15% ethanol at pH 6 and 45°C (EE), and extract produced with distilled water at pH 7 and 50°C (WE).

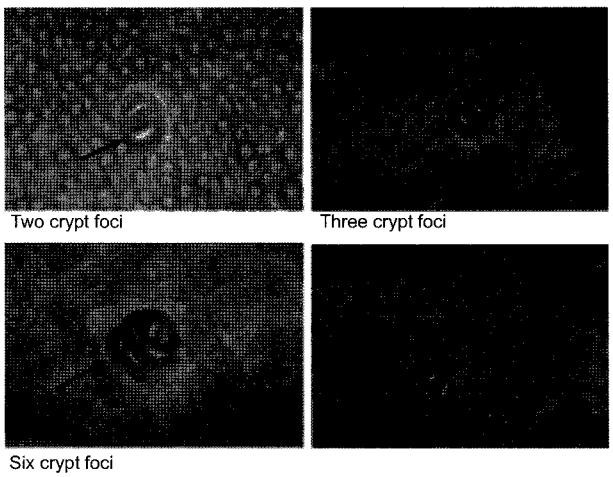

Fig. 2. Aberrant crypt foci in colonic mucosa of rats treated with 1, 2-dimethylhydrazine (DMH). Methylene blue staining (×40).

The physicochemical characteristics of oat extract influence on its physiological activities (1-5). Several physicochemical properties of oat extracts were investigated to explain the relations with their preventive activities. The chemical composition, apparent viscosity, and average molecular weight and β-(1→3):β-(1→4) bond ratios of β-glucan in these 2 extracts are listed in Table 1. EE contained 38.3% β-glucan, 13.2% starch and 25.6% crude protein, whereas WE contained 22.8% β-glucan, 35.4% starch, and 23.6% crude protein. The β-glucan content in EE was 1.6 times higher than that of WE, crude protein content was slightly higher in EE than WE, and starch content was 2.6 times higher in WE compared with EE. We previously reported that the β-glucan and crude protein contents of oat bran extracts were significantly greater with increasing amounts of ethanol and decreasing extraction temperatures, whereas the starch content showed the opposite pattern (15). The apparent viscosities, average molecular weights and β-(1 → 3):β-(1 → 4) bond ratios for β-glucan in both extracts were 8.1 and 5.9 cp, 4.6×10^6 and 4.1×10^6, and 2.52 and 2.48, respectively. The apparent viscosity, average molecular weight and β-(1→3):β-(1→4) bond ratio of EE were slightly higher than those for WE.

Body weight and daily diet intake To test the effects of each oat bran extract on the occurrence of colon cancer, rats were placed into various groups for a 6-week test period. The positive control group was fed a basal diet while injected with DMH on 4 occasions during the first 2 weeks of the test period to induce colon cancer. The remaining groups were fed a basal diet supplemented with either EE (0.25 or 0.5%) or WE (0.25 or 0.5%), with or without 4 DMH injections during the first 2 weeks. The body weights and dietary intake of rats over the 6-week test period are shown in Fig. 3 and 4, respectively. There was no significant difference in body weight and dietary intake among the experimental groups fed either EE (0.25 or 0.5%) or WE (0.25 or 0.5%), and the control groups. Dietary intake was uniform during the entire test period. Body weight and dietary intake were also similar among all groups regardless of the oat bran extract used and the dose. This suggests that the intake of oat bran extracts has no effect on the eating habits and the state of health of rats.

Table 1. Chemical composition and apparent viscosity of oat bran extracts, and the average molecular weight and β-(1→3) : β-(1 → 4) bond ratio of β-glucan in oat bran extracts

Properties	EE[1)]	WE
Chemical composition (%)		
- β-glucan	38.3	22.8
- crude protein	25.6	23.6
- starch	13.2	35.4
Apparent viscosity of 1% solution (cp)	8.1	5.9
Average molecular weigh	4.6×10^6	4.1×10^6
β-(1→3):β-(1→4) Bond ratio	2.52	2.48

[1)]Samples produced with different extraction conditions: EE, ethanol extract; WE, water extract.

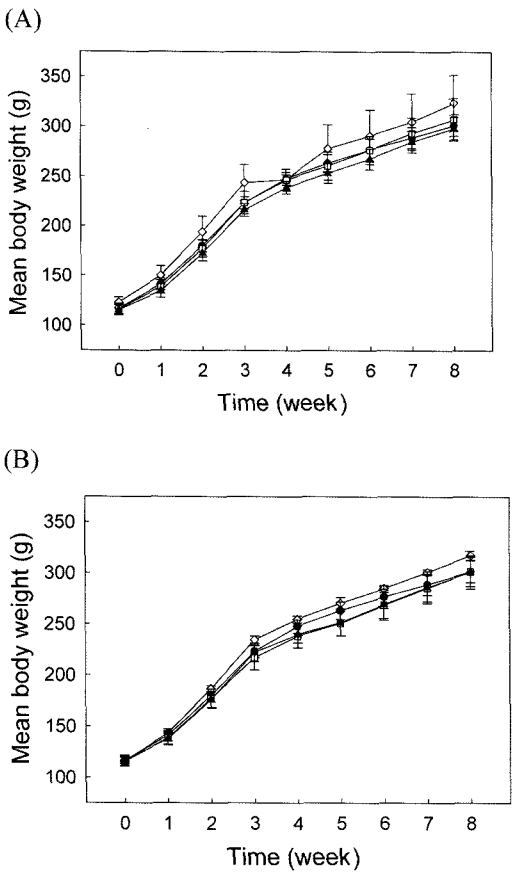

Fig. 3. Changes in body weight of rats treated with 1,2-dimethylhydrazine (DMH) followed by oat bran extract supplementation. Samples produced with different extraction conditions: (A), ethanol extract; (B), water extract. ◇ , negative control; ● , positive (DMH) control; ▲ , DMH+oat bran extract (0.25%); □ , DMH+oat bran extract (0.5%).

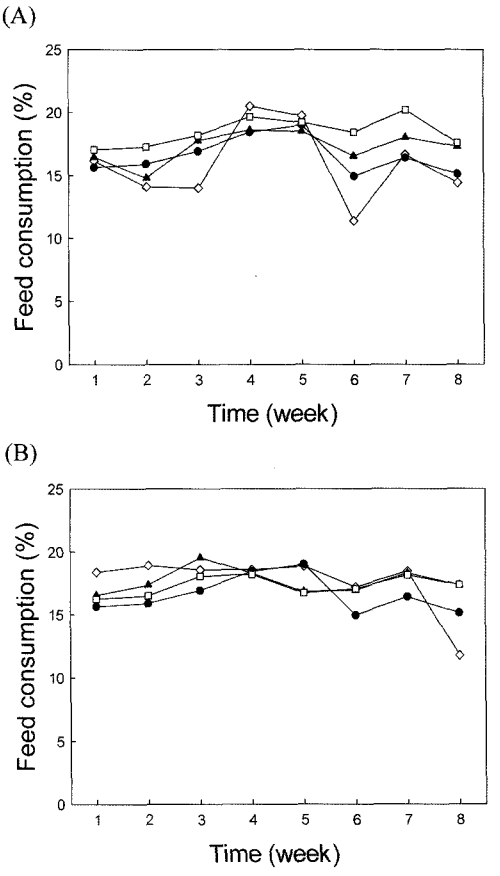

Fig. 4. Food consumption rate of rats treated with 1,2-dimethylhydrazine (DMH) followed by oat bran extract supplementation. Samples produced with different extraction conditions: (A), ethanol extract; (B), water extract. ◇, negative control; ●, positive (DMH) control; ▲ , DMH+oat bran extract (0.25%); □, DMH+ oat bran extract (0.5%)

Organ weight Liver, spleen, and kidney weights for each group are shown in Table 2. No significant differences were observed among all groups. The organ weights in the groups given 0.25 and 0.5% oat bran extracts were similar or slightly increased compared with rats given DMH alone, however this difference was not statistically significant. This indicates that neither the consumption of oat bran extract nor the injection of DMH, whether alone or in combination, leads to serious organic disorders. Evidence to support this idea comes from a previous study showing that the consumption of concentrated barley β-glucan, which has the same structure as oat β-glucan, is not associated with any obvious signs of toxicity in rats, even following the consumption of large quantities (21).

Colonic ACF The effect of oat bran extracts on the formation of AC and ACF, biomarkers for preneoplastic lesions, in rat colon cancer induced with DMH was investigated and the results are shown in Table 3. No AC or ACF were observed in rats treated with oat bran extract alone, whereas rats treated with DMH alone contained an average of 322.7 AC and 142.9 ACF per rat. With regard to the number of AC in induced ACF in the DMH alone group, an average of 228.9 AC per rat was related with 3 or less AC whereas 93.8 ACF was 4 or more AC.

Diets supplemented with oat bran extracts resulted in a marked decrease in the numbers of ACF containing 4 or

Table 2. Organ weights of rats treated with 1,2-dimethylhydrazine (DMH) followed by oat bran extract supplementation

Treatment (mg/kg)	Organ weight (g)			
	Liver	Spleen	Kidneys	
			Left	Right
DMH alone	7.26±0.68[2)]	0.61±0.06	0.90±0.07	0.88±0.07
DMH → EE[1)] (0.25%)	7.24±0.52	0.59±0.05	0.89±0.08	0.91±0.05
DMH → EE (0.5%)	7.72±0.75	0.61±0.06	0.95±0.08	0.96±0.10
EE alone (0.5%)	8.08±0.98	0.74±0.13	1.07±0.15	1.01±0.10
DMH → WE (0.25%)	7.53±0.52	0.60±0.04	0.91±0.09	0.93±0.08
DMH → WE (0.5%)	7.48±0.41	0.62±0.05	0.97±0.03	0.93±0.07
WE alone (0.5%)	7.95±0.19	0.64±0.06	0.96±0.02	0.98±0.02

[1)]Samples produced with different extraction conditions: EE, ethanol extract; WE, water extract.
[2)]Values are means±SD in each group.

Table 3. Effect of oat bran extracts on colonic aberrant crypts (AC) and aberrant crypt foci (ACF) induced by 1,2-dimethylhydrazine (DMH)

Treatment (mg/kg)	Total AC	Total ACF	Aberrant crypts	
			∑ ≤3AC	∑ ≥4AC
DMH alone	322.7±96.7	142.9±37.3	228.9±57.7	93.8±57.9
DMH → EE[1] (0.25%)	216.6±57.4	102.6±25.9	172.8±43.0	43.8±21.4*[2]
DMH → EE (0.5%)	269.6±101.2	122.0±38.5	200.1±64.1	69.5±44.8
EE alone (0.5%)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
DMH → WE (0.25%)	243.0±103.8	113.4±44.2	191.1±76.8	51.9±35.5*
DMH → WE (0.5%)	301.2±103.5	135.7±37.4	228.9±54.0	72.3±55.9
WE alone (0.5%)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

[1]Samples produced with different extraction conditions: EE, ethanol extract; WE, water extract.
[2]Significantly different from DMH alone control ($p<0.05$).

more AC. This is significant because ACF with 4 or more AC are considered to be relatively serious preneoplastic lesions with regard to the likelihood of cancer development. Interestingly, at low doses of oat bran extract (0.25 %), the total numbers of ACF with greater than or equal to 4 AC in rats given EE or WE were reduced by up to half, whereas rats given high dose oat bran extract (0.5%) showed a minor reduction that was not statistically significant. These results are in agreement with the results of a study in which the total number of ACF decreased due to a dose of 50 mg/kg body weight of 2-(allylthio) pyrazine (2-AP), yet increased at a dose of 100 mg/kg of body weight (22). We have already reported that the *in vitro* immuno-modulatory activity of these oat extracts is not necessarily related to the dose (23). This is also supported by a report of an overdose of sonifilan (β-glucan in fungus), a type of commercial anticancer drug, reducing or eliminating its anticancer effect toward S-180 cells. The basis of this is that excess sonifilan inhibits TNF-α (tumor necrosis factor- α) production and macrophage adherence (24). This implies that a high dose of β-glucan may retard or inhibit the anticancer effect by hindering immunomodulatory activities. Therefore, it appears necessary to determine the ideal dose of β-glucan or oat bran extract through additional experimentation to maximize its suppression of colon cancer.

On the other hand, it has been reported that the acidification of colonic contents by high fiber diets fails to inhibit rat colon carcinogenesis. Oat bran consumption has also been associated with the enhancement of proximal colon carcinogenesis after DMH was injected at 20 mg/kg body weight on a weekly basis for 12 weeks (25). This is probably due to the use of excessively high doses of carcinogen resulting in high tumor yields which respond to the proliferative effects of dietary fiber and thus enhance the carcinogenic process. This suggests, comparing with other report (25), that the dose of carcinogen (DMH) in this model system is optimal level, which is able to explain the preventive effect of oat bran extract on colon cancer. It has also been proposed that the protective activity of crude β-glucan from oat bran on colon cancer is more effective during the initiation process of carcinogenesis. Carcinogenesis is a complex and multistep process beginning at the cellular level. The whole process can be divided into the three main stages of initiation, promotion, and progression. Chemical carcinogens, such as DMH, azoxy-methane (AOM), and 7,12-dimethylbenz[a]-anthracene (DMBA), are changed into electrophiles through metabolic processes catalyzed by oxidation enzymes such as cytochrome P-450 oxidoreductases (CYP enzymes) that by themselves have no carcinogenicity. These electrophiles produce DNA adducts resulting from their reaction with DNA in the living body (26). If this DNA injury is not repaired carcinogenic mutations can be propagated by the process of DNA replication. Such cells exposed continuously to carcinogenic factors progress from the initiation stage to the promotion stage and therefore a relatively benign lesion can become malignant and develop into a fast growing neoplasm (27). It is known that the enhancement of epithelial cell division in the human large bowel correlates with the incidence of colon cancer, and that the formation of AC resulting from cell proliferation produces the preneoplastic lesions of colon cancer (28, 29). Specifically, it was reported that ACF with 4 or more AC showed a high correlation with the incidence of tumor formation (30). Therefore, the initial prevention of carcinogenesis may be very important, which makes the results of the present study especially significant.

With regard to the number of ACF in two oat bran fractions tested in this study, there was no significant difference. Considering that the modulation of small intestinal mucosal structure and growth by fiber appears to depend on the quantity and quality of the fiber (7), this result seems to correlate with the difference of β-glucan content in each fraction. However, several minor differences in the characteristics of each extract, including viscosity, molecular weight, β-(1→3):β-(1→4) bond ratio and so on, may account for their differing effects. Specifically, when compared to WE, the higher β-glucan and protein content, slightly higher viscosity, average molecular weight and β-(1 → 3):β-(1 → 4) bond ratio in EE might account for the decreased numbers of AC or ACF. It may be possible to partially explain this from the fact that the high viscosity and molecular weight of β-glucan can enhance the preventive effect by improving carcinogen absorption and removal, and that a high β-(1 → 4) bond ratio can increase the insolubility of β-glucan and thus increase stool weight, diluting the carcinogens (1, 5). However, preventive effects based on

the characteristics of β-glucan and other components in the extracts are difficult to explain based solely on this experimental data. Additional experiments with purified oat β-glucan or characterized oat β-glucan (i.e., fractiona-tion by MW and β-(1→3):β-(1→4) bond ratio, etc.) are necessary to specifically assess these effects.

In conclusion, the present study demonstrates that oat bran extracts can provide health benefits with regard to the prevention of colon cancer by reducing the total number of preneoplastic ACF with 4 or more AC at a relatively low dose with no obvious sign of toxicity in rats.

Acknowledgments

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