

# Preventive effect of fermented brown rice and rice bran against colon carcinogenesis in male F344 rats

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**Abstract.** Epidemiological and preclinical studies demonstrate that nutrition plays an important role in the etiology of cancer. It has been reported that rice components, especially rice germ plays a key role in prevention of cancer. The experiments described here examined the potential anticancer properties of brown rice fermented by *Aspergillus Oryzae* (FBRA) in male F344 rats using inhibition of the formation of azoxymethane (AOM) induced aberrant crypt foci (ACF) and tumors in the colon as the measure of preventive efficacy. The agent was administered at 2.5 and 5% levels in the diet during the initiation phase (during and until 1 week after carcinogen treatment) and/or post-initiation phase (beginning 1 week after carcinogen treatment) of carcinogenesis. In the ACF and tumor studies, rats were sacrificed 5 or 40 weeks after the initiation of AOM treatment (15 mg/kg body weight, once weekly for 3 weeks), respectively. Colonic ACF and tumors were evaluated histopathologically. Administration of 2.5 and 5% FBRA in the diet continuously during initiation and post-initiation period significantly inhibited the ACF formation in rats treated with AOM, compared with rats treated with AOM alone ( $99 \pm 24.1$  and  $79 \pm 18.4$  vs.  $139.5 \pm 27.7$ , respectively). In addition, administration of 5% FBRA in the diet during the post-initiation phase significantly suppressed the incidence (44 vs. 18%) and

multiplicity ( $0.93 \pm 0.96$  vs.  $0.18 \pm 0.40$ ) of colon adenocarcinomas as compared to those given the control diet. In addition, 5% FBRA in the diet during post-initiation phase caused significant inhibition of cell proliferation in the colonic mucosa as compared to the group fed the control diet (81% reduction,  $p < 0.05$ ). These observations demonstrated for the first time that FBRA inhibits colon tumor development in rats, and suggest that it is a promising dietary supplement for prevention of human colon cancer.

## Introduction

It has been recognized that dietary factors play an important role in the prevention of several types of cancer including cancer of the colon. Epidemiological studies have shown that high intake of fruit, vegetables and cereal foods decrease the risk, whereas high intake of dietary fat increases risk of colon cancer incidence (1,2). Preclinical studies have also provided evidence that several components of these foods reduce the risk of colon cancer (3-7). Rice is one of the major cereal foods eaten as the staple food worldwide, and especially in Asian countries. Rice seeds and rice germ contain fiber (8) and several kinds of antioxidants, such as ferulic acid (9), phytic acid (10), tocopherols and oryzanols (11). Among them, fiber (8), phytic acid (12), and ferulic acid (13,14) have been reported to prevent chemically-induced carcinogenesis in preclinical models. There has been growing interest in the prevention of colon cancer and other types of cancer by rice-based and other types of cereal grains in view of the observations that these foods contain high amount of fiber, several antioxidants and related compounds, which are believed to be largely responsible for their anticancer properties. Importantly, the studies conducted in our laboratory demonstrated that rice germ has an inhibiting effect on AOM induced colon carcinogenesis (15), suggesting that brown rice (unpolished rice) which contains several of these compounds possesses potential chemopreventive properties. FBRA is a processed food prepared by fermenting brown rice and rice bran with *Aspergillus Oryzae*. It is already known that FBRA acts as a potent free radical scavenger (16). The significance

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*Abbreviations:* AOM, azoxymethane; FBRA, fermented brown rice; ACF, aberrant crypt foci; PCNA, proliferation cell nuclei antigen

*Key words:* fermented brown rice, rice bran, colon cancer, cell proliferation

of brown rice and rice germ as modulators of carcinogenesis prompts us to explore FBRA for its potential anticancer properties.

ACF are recognized as putative preneoplastic lesions that occur in the colon of both animals and humans (17,18). Studies by Bird (17) have documented the presence of ACF in the colon of rodents after treatment with a colon carcinogen. Pretlow *et al* (18) reported the presence of putative preneoplastic lesions in normal-appearing mucosa of patients with colon cancer. Multiplicity of ACF increases overtime and reliably to be a predictor of colon tumor outcome (18). There is evidence that several inhibitors of ACF development reduce colon tumorigenesis in laboratory animals (19). The present study was designed to investigate possible preventive effect of dietary FBRA administered during the initiation and/or post-initiation stage of AOM-induced ACF formation and development of tumors in the colon. We also examined the effect of FBRA on cell proliferation activity in the colonic epithelium by analysis of proliferation cell nuclei antigen (PCNA) positive index. The ultimate goal of this study was to examine whether FBRA is an effective food supplement against chemically-induced colon carcinogenesis in a well-established preclinical model and, eventually, in human clinical trials.

## Materials and methods

**Animals, diets, carcinogen and FBRA.** Male F344 rats (Shizuoka Laboratory Animal Center, Shizuoka Japan) received at 4 weeks of age were housed in wire cages (3 or 4 rats/cage) under controlled conditions of humidity (50-60%), lighting (12-h light/dark cycle) and temperature ( $23\pm 2^{\circ}\text{C}$ ), with free access to water and a basal diet CE-2 (CLEA Japan, Tokyo). They were quarantined for 2 weeks, and then randomized into experimental and control groups. Powdered CE-2 diet was used as the basal diet throughout the study. The experimental diets were prepared by mixing 1.25, 2.5 and 5.0% FBRA with CE-2 diet, respectively.

**Chemicals.** AOM was purchased from Sigma chemical Co. (St. Louis, MO). FBRA was supplied by Genmai koso Co., Ltd. (Sapporo, Japan). Briefly, the manufacturing process of FBRA is as follows. Fermentation base was made by steaming of brown rice and rice bran. *Aspergillus Oryzae* was then seeded to the fermentation base and fermentation process was continued for 18-24 h. Subsequently, second fermentation was continued for additional 12-24 h for aging purpose. Fermented product was then dried and powdered. Final composition of FBRA is shown in Table I.

**Experimental procedure.** The experimental design is summarized in Fig. 1. For ACF study, a total of 56 male F344 rats were divided to 5 groups (Fig. 1A). At 6 weeks of age, the rats in groups 1-4 (12 rats in each group) intended for carcinogen treatment received AOM, at a dose rate of 15 mg/kg b.w., s.c. once weekly for 2 weeks, whereas animals in group 5 received an equal volume of normal saline and served as vehicle controls. Animals in groups 2, 3 and 4 were fed the FBRA at levels of 1.25, 2.5 and 5.0% respectively in the basal diet throughout the experimental period (from 5 to 11 weeks

Table I. Composition of FBRA.

	Amount/100 g FBRA
Hydrosis	3.3 g
Protein	23.8 g
Fat	20.5 g
Ash	9.0 g
Hydrocarbon	22.4 g
Fiber	21.0 g
Pytic acid	3.86 g
Vitamin A	0.03 mg
Vitamin B group	4.97 mg
Vitamin E group	15 mg
Vitamin K group	63 mg
Sodium	8.1 mg
Phosphorus	1.96 g
Calcium	308 mg
Iron	9.9 mg
Magnesium	810 mg
Copper	936 mg
Zinc	5.99 mg
Manganese	15.6 mg
Selenium	8 $\mu\text{g}$
Alabinoxylane	5.6 g

of age), whereas animals in group 5 who received normal saline was administered 5% FBRA in the diet. Body weights were recorded weekly until termination of the study. At 11 weeks of age, all animals were sacrificed by decapitation and the colons were cut open longitudinally, washed with normal saline, and fixed in 10% buffered formalin for 24 h and then stained with 0.5% methylene blue for analysis of ACF. ACF were recorded according to procedure of Bird (17). ACF were distinguished by their increased size, their more prominent epithelial cells and their increased pericryptal space compared with surrounding normal crypts. The number of ACF per colon and the number of aberrant crypts per colon were recorded. The parameters used to assess the aberrant crypts were their occurrence and multiplicity. Crypt multiplicity was determined as the number of crypt in each focus.

For tumor study, a total of 182 rats were divided into 7 groups (Fig. 1B). The rats in groups 1-5 received s.c. injection of AOM (15 mg/kg body weight) once a week for 3 weeks beginning at 5, 6 and 7 weeks of age. Whereas animals in group 6 and 7 received normal saline and served as vehicle controls. The rats of group 2 and 3 were administered FBRA at 2.5 and 5% in diet, respectively, during initiation stage (during and until 1 week after AOM treatment) and then transferred to the control diet until the termination (for

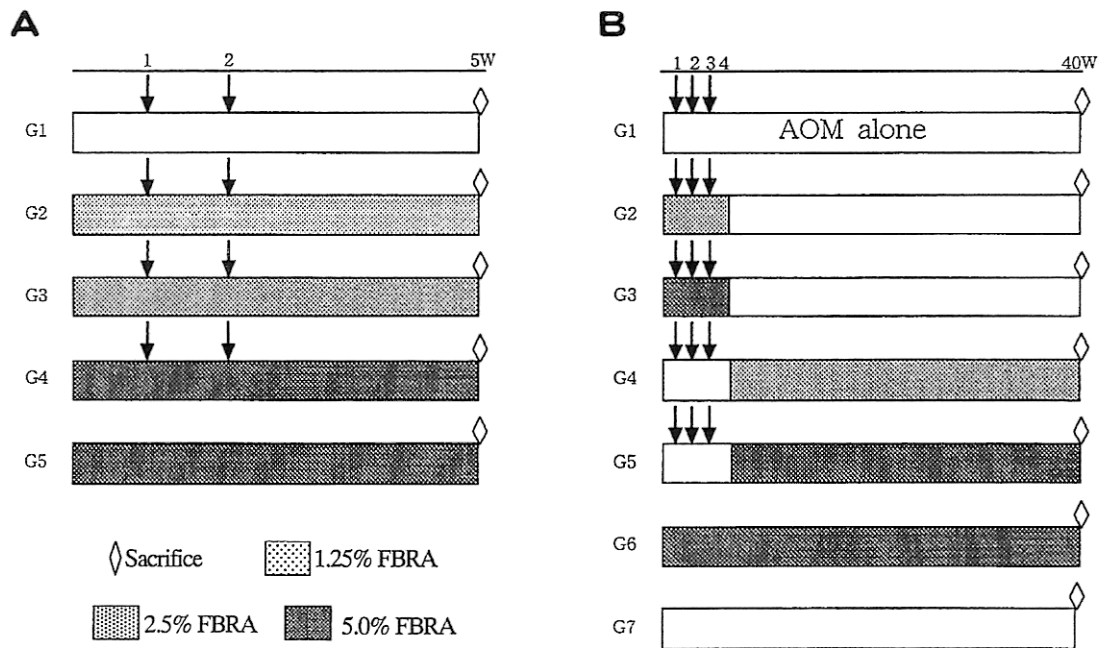


Figure 1. Experimental design to study the effect of the fermented brown rice on colon carcinogenesis. (A), ACF study. (B), colon tumor study. ↓, AOM s.c. injection (15 mg/kg body weight).

37 weeks after the last AOM treatment). Animals in groups 4 and 5, which were on basal diet during the carcinogen treatment, received 2.5 and 5% FBRA in diet, respectively, during the post-initiation phase (1 week after the third AOM treatment) whereas animals in group 6 were fed on 5% FBRA throughout the experimental period. Rats in group 7 which received control diet throughout the study period served as non-treatment control. All animals in each group were weighed once a month. All rats were sacrificed by decapitation at the end of study. At autopsy, livers were excised and weighed. Intestines were opened longitudinally and contents were flushed with normal saline. They were examined for the presence of tumors. Colon and small intestinal tumors were fixed in 10% buffered formalin and processed for histopathological examination by routine procedure. Intestinal neoplasms were diagnosed according to the criteria described by Ward (20). Abnormal lesions in other organs were also examined histopathologically.

**PCNA immunohistochemistry.** PCNA immunohistochemistry was performed in tumor-free colonic mucosa in order to assess the proliferate activity of colonic mucosal cells. The immunohistochemical stains were performed according to a previously published method (21). Anti-PCNA antibody and LSAB kit (Dako Co., Kyoto, Japan) was used for PCNA immunohistochemical stain. To determine PCNA positive index, 15 full-length crypts from each colon in each of 10 rats from group 1-5 (Fig. 1B) were randomly examined. Numbers of positively stained nuclei were counted and divided by the total number of nuclei to determine the PCNA-positive index (22).

**Statistical analysis.** Differences in tumor incidences among the rats fed the diets containing FBRA were compared by

Fisher's exact probability, and differences in body weight and liver weights were analyzed by one-factor ANOVA and Dunnett's method for multiple comparisons to determine the significance among each group. ACF and tumor multiplicities and PCNA positive cell index were analyzed by Student's t-test or Welch's t-test. The results were expressed as mean  $\pm$  SD. A value of  $p < 0.05$  was considered as significant.

## Results

The body weight and liver weight of the rats treated with vehicle or with AOM and fed either control or experimental diets containing different levels of FBRA during the initiation stage were comparable throughout the study whereas the body weights of rats fed the diets containing 2.5 and 5.0% FBRA were significantly higher than the control diet group (data not shown). The effect of FBRA on AOM-induced ACF development is shown in Table II. AOM treatment, on average induced about 140 ACF/colon and 266 total aberrant crypts/colon in rats on the control diet. Administration of FBRA at 2.5 and 5.0% levels significantly suppressed the total number of ACF/colon ( $p < 0.05$ ) and the total number of aberrant crypts/colon ( $p < 0.05$  and  $0.01$ ). The incidence of multicrypt aberrant foci (2 crypt/ ACF) was also suppressed in 5.0% FBRA diet group ( $p < 0.01$ ). The results summarized in Table III indicate that administration of 5% FBRA in the diet during the post-initiation stage significantly inhibited the incidence of colonic adenocarcinomas and total tumors in the colon and small intestine when compared with the results of the rats fed the control diet. Moreover, the multiplicity of total tumors in the colon and small intestine was significantly lower in animals fed the 2.5 and 5.0% FBRA during the post-initiation period when compared with the results for rats fed

Table II. Effect of FBRA administered during the initiation and post-initiation stage on the development of AOM-induced ACF.

Group	Treatment	Total no. of ACF/colon	No. of ACF containing				Total aberrant crypts
			1 crypt	2 crypts	3 crypts	4 or more crypts	
1	AOM alone	139.5±27.7 <sup>a</sup>	51.8±14.8	57.8±13.1	22.5±6.3	1.8±3.0	266.1±55.7
2	AOM + 1.25% FBRA	122.6±27.7	40.1±11.9	52.1±8.5	22.3±8.1	2.0±3.2	245.5±62.0
3	AOM + 2.5% FBRA	99±24.1 <sup>c</sup>	31.8±8.2 <sup>c</sup>	39.7±9.3 <sup>c</sup>	20.4±9.3	1.8±3.5	202.6±61.2 <sup>b</sup>
4	AOM + 5% FBRA	79±18.4 <sup>c</sup>	23.9±0.7 <sup>c</sup>	33.0±8.4 <sup>c</sup>	18.4±5.9	0.9±1.6	160.4±37.6 <sup>c</sup>
5	5% FBRA alone	0	0	0	0	0	0

<sup>a</sup>Mean ± SD. <sup>b,c</sup>Significant difference from group 1 by Student's t-test or Welch's t-test (<sup>b</sup>p<0.05, <sup>c</sup>p<0.01).

Table III. Effect of FBRA on AOM-induced intestinal tumor incidence in male F344 rats.

Group	Treatment <sup>a</sup>	No. of rats	Intestine including colon and small intestine			Colon		
			AD (%) <sup>b</sup>	ADC (%)	Total (%)	AD (%)	ADC (%)	Total (%)
1	AOM + basal diet	27	9 (33)	15 (56)	19 (70)	8 (30)	12 (44)	18 (67)
2	AOM + 2.5% FBRA	24	6 (25)	11 (45)	14 (58)	4 (18)	9 (38)	12 (50)
3	AOM + 5.0% FBRA	27	7 (26)	15 (56)	17 (63)	6 (22)	14 (42)	16 (59)
4	AOM→2.5% FBRA	26	5 (19)	12 (46)	16 (59)	4 (15)	9 (35)	12 (46)
5	AOM→5.0% FBRA	28	6 (21)	6 (21) <sup>c</sup>	11 (39) <sup>d</sup>	4 (14)	5 (18) <sup>c</sup>	8 (29) <sup>c</sup>
6	5% FBRA	15	0	0	0	0	0	0
7	Basal diet alone	16	0	0	0	0	0	0

<sup>a</sup>In groups 2 and 3, FBRA diet was fed during the initiation stage whereas in groups 4 and 5, FBRA diet was fed during the post-initiation stage.

<sup>b</sup>AD, adenoma; ADC, adenocarcinoma; total, adenomas + adenocarcinomas. <sup>c,d</sup>Significantly different from control group by Fisher's exact probability (<sup>c</sup>p<0.05, <sup>d</sup>p<0.01).

Table IV. Effect of FBRA on AOM-induced intestinal tumor multiplicity in male F344 rats.

Group	Treatment	No. of rats	Intestine including colon and small intestine			Colon		
			AD	ADC	Total	AD	ADC	Total
1	AOM + basal diet	27	0.44±0.85 <sup>a</sup>	0.85±1.03	1.30±1.14	0.33±0.55	0.67±0.96	1.00±0.92
2	AOM + 2.5% FBRA	24	0.25±0.44	0.63±0.77	0.88±0.95	0.17±0.38	0.50±0.72	0.67±0.82
3	AOM + 5.0% FBRA	27	0.30±0.54	0.89±1.05	1.19±1.30	0.22±0.42	0.67±0.73	0.89±0.85
4	AOM→2.5% FBRA	26	0.19±0.40	0.46±0.51	0.65±0.56 <sup>b</sup>	0.15±0.37	0.35±0.49	0.50±0.58 <sup>b</sup>
5	AOM→5.0% FBRA	28	0.29±0.61	0.21±0.42 <sup>c</sup>	0.50±0.75 <sup>b</sup>	0.18±0.48	0.18±0.40 <sup>c</sup>	0.36±0.63 <sup>c</sup>
6	5% FBRA	15	0	0	0	0	0	0
7	Basal diet alone	16	0	0	0	0	0	0

<sup>a</sup>In groups 2 and 3, FBRA diet was fed during the initiation stage whereas in groups 4 and 5, FBRA diet was fed during the post-initiation stage.

<sup>b</sup>AD, adenoma; ADC, adenocarcinoma; total, adenomas + adenocarcinomas. <sup>c</sup>Mean ± SD. <sup>d,e</sup>Significant difference from group 1 by Student's t-test or Welch's t-test (<sup>d</sup>p<0.05, <sup>e</sup>p<0.01).

Table V. Effect of dietary FBRA on PCNA positive cell indices in the colonic crypts.

Group	Treatment	No. of rats	PCNA positive cell indices
1	AOM alone	10	29.1±2.3 <sup>b</sup>
2	AOM + 2.5% FBRA	10	26.1±3.3 <sup>c</sup>
3	AOM + 5% FBRA	10	25.8±3.4 <sup>c</sup>
4	AOM→2.5% FBRA	10	25.6±4.4 <sup>c</sup>
5	AOM→5% FBRA	10	23.7±3.4 <sup>d</sup>

<sup>a</sup>In groups 2 and 3, FBRA diet was fed during the initiation stage whereas in groups 4 and 5, FBRA diet was fed during the post-initiation stage. <sup>b</sup>Mean ± SD. <sup>c,d</sup>Significant difference among all groups by Student's t-test or Welch's t-test (<sup>c</sup>p<0.05, <sup>d</sup>p<0.01).

the control diet. Adenocarcinomas in colon and small intestine were significantly lower in 5.0% FBRA group. However, there were no significant differences in the incidences and multiplicities of colon and total intestinal tumors among the animals fed the FBRA and control diets during the initiation stage. The effect of dietary FBRA on cell proliferation is summarized in Table V. PCNA positive cell indices of groups 2-5 (26.1±3.3, 25.8±3.4, 25.6±4.4, and 23.7±3.4, respectively) were rather less than that of group 1 (29.1±2.3). Administration of 5% FBRA in the diet significantly reduced PCNA positive cell index compared with the group fed the control diets (p<0.05).

## Discussion

This study was undertaken to evaluate the efficacy of FBRA on the formation of chemically-induced colonic preneoplastic lesions and tumors in a well-established preclinical model. Previous studies from our laboratory indicate that rice germ inhibit colon carcinogenesis (15). In the current study, we have evaluated the efficacy of dietary FBRA against colon carcinogenesis using ACF and tumors as endpoints. The data presented demonstrate that compared with a control diet, administration of 5% FBRA in the diet during the initiation and post-initiation phase significantly suppressed not only the total number of ACF but also total number of aberrant crypts in the colon. Recent studies suggest that there is a high degree of correlation between the ACF formation and outcome of colon tumors at the later stages. Thus the present study shows that administration of 5% FBRA diet significantly inhibits the formation and growth of preneoplastic lesions. The present study also demonstrated for the first time that the dietary FBRA at 5% level administered during the post-initiation phase strongly inhibits the incidence and multiplicity of colon adenocarcinomas in a well-established preclinical model of colon carcinogenesis. This findings underscores the likelihood that FBRA may be an effective food-derived

supplement against colorectal cancer. In this study, index of PCNA positive cells in the colonic mucosa of rats in experimental groups given FBRA during post-initiation phase were significantly smaller than of the group with carcinogen alone. Similar results have been obtained in other studies with different cancer preventive agents (23). Suppression of carcinogen-induced hyper-proliferation of cells in the target organs regarded as important mechanisms for the chemopreventive agents (24,25).

It is known that the rice components have been shown to prevent several chronic diseases including several types of cancer (26). Recently, it has been reported that inositol hexaphosphate (IP6), which is a constituent of rice bran, most cereals, nuts, oilseeds, legumes, has been reported to reduce incidence of carcinogen-induced large bowel cancer (11) and inhibit growth of transplanted tumors (27). Rice bran contains approximately 20% oil which contains several bioactive polyphenols including ferulic acid with anti-oxidantive properties. We have reported earlier a potential chemopreventive effect of ferulic acid against AOM-induced colon carcinogenesis (13) and 4-nitrosoquinoline-1-oxide-induced tongue carcinogenesis in rats (23,28).

Brown rice is not well accepted by current generation of people who have been accustomed to white milled rice as daily staple food. It should be noted that in the current study major component of FBRA is brown rice and rice bran that received fermentation by *Aspergillus Oryzae*. The advantage of fermentation has been recognized in recent years, but the details are not well known. Japanese traditional fermented soybean products, like soybean paste (miso) or soy sauce (syoyu), have been more stable against lipid peroxidation than unfermented soybean. Esaki *et al* (29) reported that soybean products fermented with *Aspergillus Oryzae* contains antioxidant, such as 6-hydroxydaidzein, 8-dehydroxydaidzein, and 8-hydroxygenistein. Preventive effects of soybean products have been shown in recent epidemiological (30) and experimental studies (31,32). However little is known about the efficacy of fermented rice. The present study is the first to determine the preventive efficacy of fermented rice. The precise mechanisms by which FBRA inhibits colon carcinogenesis is not fully known, yet it is likely that the efficacy of FBRA is mediated through the action of several trace elements including selenium which is biotransformed to organic form during the fermentation of brown rice and rice bran (33). Kurosu which is produced from brown rice through stationary surface acetic acid fermentation, is reported to have antioxidant properties (34). It is also possible that the antioxidant activity of FBRA may be associated with the suppression of colon carcinogenesis in the current study. Further studies are needed to understand the mechanism by which dietary FBRA suppresses colon carcinogenesis.

In conclusion, our findings with fermented brown rice and rice bran that has been evaluated for the first time as preventive agent against colon carcinogenesis, have shown that this food supplement significantly suppresses the incidence of colonic ACF and tumorigenesis in male F344 rats. Also, FBRA significantly inhibited the colonic mucosal cell proliferation. While understanding of the mechanisms of FBRA is evolving, our data imply that dietary FBRA can potentially assist in reducing human colorectal cancer.

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