

Preventive effect of fermented brown rice and rice bran on *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced gastric carcinogenesis in rats

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Abstract. A number of possible preventive agents for cancers in different organs have been reported, however, little information is available regarding the effective agents for the development of gastric cancers. The rice components are known to be effective for the prevention of the development of cancers. Our group has demonstrated that fermented brown rice by *Aspergillus Orzae* (FBRA) has chemopreventive potentials in several organs. In this study, we investigated the modifying effects of FBRA exposed during the initiation or post-initiation phase of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-induced gastric carcinogenesis in rats. Five-week-old male ACI rats were divided into 7 groups. Groups 1-5 were given oral administration of MNNG (100 mg/l in distilled water) for 24 weeks starting at 6 weeks of age. Groups 2 and 3 were fed a diet containing 5 and 10% FBRA during the initiation phase, respectively, whereas groups 4 and 5 were fed these diets during the post-initiation phase. Group 6 was given a diet containing 10% FBRA throughout the experiment. Group 7 was kept on the basal diet alone and served as an untreated control. Rats were sacrificed at 52 weeks after the start, and the epithelium of the stomach was investigated in detail. Incidence and multiplicity of gastric proliferative lesions of group 1 (MNNG alone) were 61% and 1.67 ± 1.57 /rat, respectively. Those of group 5 (25%, 0.35 ± 0.67) which were given FBRA at a dose of 10% during the post-initiation phase were significantly less than those of group 1. Furthermore, the same group expressed a significantly

decreased Ki67-labeling index in the non-lesional gastric epithelium when compared to that of group 1. These results indicate that FBRA inhibits MNNG-induced development of gastric tumors by administration during the post-initiation phase in rats. FBRA is regarded as a promising dietary agent for the prevention of human gastric cancer.

Introduction

Gastric cancer is the fourth most common cancer and the second most common cause of cancer deaths worldwide (1,2). Notwithstanding the global declining incidence of gastric cancer, mortality is still rising in Asian countries. Countries with a particularly high incidence of this disease include Japan, China and Korea. Most epidemiological studies show that a high intake of smoked, salted and nitrated foods, carbohydrates and a low intake of fruits, vegetables and milk significantly increase the risk of gastric cancer (2-4). It is suggested that the dietary consumption plays an important role in the development of gastric cancer, and natural products in traditional diets exert protective activities against the development of gastric cancers.

Rice is one of the major cereal foods eaten as a staple food worldwide, especially in Asian countries. Rice seeds and rice germ contain fiber and several kinds of antioxidants, such as ferulic acid (5), phytic acid (6), tocopherols and oryzanols (7). Among them, fiber (8) and ferulic acid (9,10) have been reported to prevent carcinogen-induced aerodigestive tract carcinogenesis in animal models. We have reported chemopreventive effects of rice germ itself or the compounds contained in the rice bran or germ against carcinogenesis in the large bowel or tongue of rodents (11,12). Fermented brown rice by *Aspergillus Orzae* (FBRA) is a processed food prepared by fermenting brown rice and rice bran with *Aspergillus Orzae*. We previously reported that FBRA has inhibitory effects on the carcinogenesis of the colon, liver, esophagus and urinary bladder in rodents (9,13-15). It is already known that FBRA has antioxidative activity and is

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regarded as an important mode of action of chemopreventive agents (16).

The method using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) for the induction of stomach cancers in rodents is considered to be a good model of human gastric cancers (17,18). In the present study, we examined the chemopreventive potential of FBRA on MNNG-induced gastric carcinogenesis in rats. In addition, we measured cell proliferation and apoptosis in the stomach epithelium for an understanding of functional mechanisms of FBRA.

Materials and methods

Animals, diets, carcinogen and FBRA. Male ACI rats, 4 weeks old, were purchased from Japan SLC, Inc. (Hamamatsu). After 1 week of quarantine, rats were transferred to the holding room under controlled conditions at $23\pm 2^{\circ}\text{C}$ temperature, $50\pm 10\%$ humidity, and a 12-h light/dark cycle. They were housed in wire cages (3 or 4 rats/cage). Powdered CE-2 (CLEA Japan, Inc., Tokyo) was used as the basal diet for the experiment. MNNG was obtained from Tokyo Chemical Inc. (Tokyo, Japan). The experimental diets were prepared by mixing 5 or 10% FBRA with CE-2 diet. The diets were stored in a cold room (4°C) and were freely available to the animals. FBRA was supplied by Genmai Koso Co., Ltd. (Sapporo, Japan). The manufacturing process of FBRA and its composition was reported previously (9).

Experimental procedure. The experimental design was approved by the Institutional Ethics Review Committee for animal experiments at the Gifu University. A total of 147 rats were randomized into 7 groups as shown in Fig. 1. At 6 weeks of age, rats in groups 1-5 received MNNG in drinking water for 24 weeks. MNNG (Tokyo Chemical) was dissolved in distilled water at a concentration of 100 mg/l and freshly prepared thrice per week for administration in drinking water in light-shielded bottles *ad libitum*, according to the protocol described in previous reports (19-21). Rats in groups 2 and 3 were given a diet containing 5 and 10% FBRA for 26 weeks, respectively, starting one week before MNNG exposure until the end of week 26. They were switched to and maintained on the basal diet until termination. Starting 1 week after the cessation of MNNG treatment, rats in groups 4 and 5 were fed a diet mixed with 5 and 10% FBRA, respectively, which continued until termination. Rats in group 6 were fed a diet containing 10% FBRA during the experiment. Rats in group 7 were given the basal diet and tap water throughout the experiment and served as controls. All rats were carefully inspected daily, and consumption of the experimental diets mixed with the test compound was recorded to estimate dietary intake. Fifty-two weeks after it began, the experiment was terminated.

Tissue preparation and histopathological examination. At the end of the experiment, all rats were sacrificed to evaluate the frequency of preneoplastic and neoplastic lesions in the stomach. During the autopsy, the stomach of all the rats were inflated with 10% buffered formalin, fixed overnight in 10% buffered formalin, bisected longitudinally, inspected for gross lesions, then embedded in paraffin for a histopathological

evaluation on hematoxylin and eosin (H&E)-stained sections. The defining characteristics for atypical hyperplasia and adenocarcinoma were adapted from previous literature (22). Atypical hyperplasia were characterized by glandular proliferation with mild structural and cellular atypia, intramucosal adenocarcinomas by excessive glandular proliferation with pronounced structural and cellular atypia within the mucosa, and invasive adenocarcinomas by cellular atypia and atypical glandular structures with invading at least the submucosa. Other macroscopically abnormal organs were examined histologically. The liver and kidney were fixed with 10% formalin, embedded in paraffin blocks and processed routinely for a histopathological examination.

Immunohistochemistry. The avidin-biotin peroxidase complex (ABC) technique was used for immunohistochemical studies. Five- μm thick sections were cut, deparaffinized, rehydrated in PBS, placed in 10 mmol/l citrate buffer (pH 6.0), and heated in a 750-W microwave four times for 6 min. The endogenous peroxidase activity was blocked by incubation for 30 min in 0.3 H_2O_2 . After washing three times with PBS, the sections were preincubated with a normal blocking serum for 20 min at room temperature and then incubated with Ki67 (Dako, Carpinteria, CA, #M7248; 1:200) and cleaved caspase-3 (Cell Signaling, Danvers, MA, #9661; 1:200) overnight at 4°C . Subsequently, the sections were incubated with biotinylated secondary antibodies (Vectastain ABC kit, Vector Laboratories) for 30 min, followed by incubation with avidin-coupled peroxidase (Vector Laboratories) for 30 min. The sections were developed with 3,3'-diaminobenzidine (DAB) using Dako liquid DAB substrate-chromogen system (Dako Corp.) and were then counterstained with hematoxylin. The specificity of the binding was confirmed by omitting the primary antibody, and this staining was used as a negative control.

Cell proliferation and apoptosis analysis. Cell proliferation index in non-lesional lesions of the stomach was determined by immunohistochemistry with a Ki67 antibody. Cells undergoing apoptosis were determined by immunohistochemistry with activated cysteinyl aspartic acid-protease-3 (cleaved caspase-3) antibody. The percentage of Ki67- and cleaved caspase-3-positive cells, i.e., the labeling index in gastric gland tissues were determined by two persons in a blinded manner regarding the conditions of the section. An average of 500 cells were counted for each section stained for the detection of either Ki67- or cleaved caspase-3-positive cells under $\times 400$ magnification at light microscopy.

Statistical analysis. Statistical analysis of the incidence of gastric lesions was performed using the Fisher's exact test. The data on the multiplicity of gastric lesions, Ki67 index and apoptosis index were examined by using the Student's *t*-test.

Results

General observation of experimental animals. Rats from groups 1-7 tolerated well the oral administration of MNNG. There were no significant differences in the mean intake of MNNG among groups 1-5. The mean intake of food was

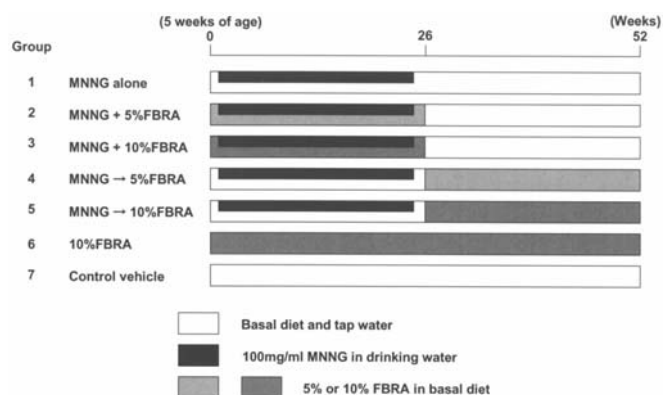


Figure 1. Experimental design. A total of 147 rats were used in the experiment. All rats were carefully autopsied at the time of their death, either after having been sacrificed because they had become moribund or at the end of the experiment (week 52).

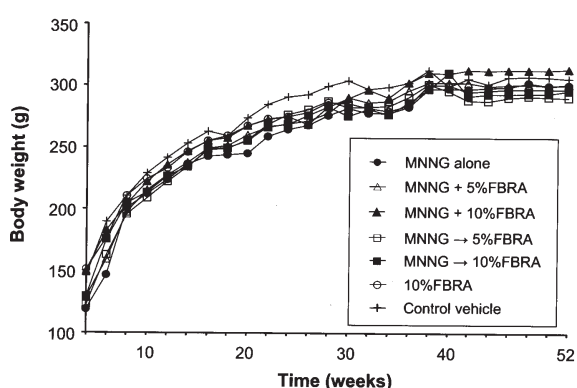


Figure 2. Body weight of animals in the different treatment groups during the experiment. There was no significant difference in body weight among the groups.

constant and similar among the different groups during the entire treatment period. There was no significant difference in body weight among all groups during the entire study period (Fig. 2). In addition, there were no marked differences in liver, or kidney weight among the groups (data not shown). There were no toxicopathological findings in the liver and kidney of any rat examined. There were in total 8 deaths during the study period, which were due to gastrointestinal cancer (n=4) or non-digestive tract diseases (n=4).

Effect of FBRA on glandular stomach carcinogenesis. The gastric tumors were solitary or multiple lesions and developed in the glandular stomach, predominantly in the antrum (Fig. 3). Histologically, these tumors were atypical hyperplasia or adenocarcinomas. In addition, several spindle sarcomas were developed in the glandular stomach. The incidence and multiplicity (number of tumors/rat) of gastric tumors are summarized in Tables I and II, respectively. Atypical hyperplasia was classified as a benign tumor, whereas adenocarcinoma and sarcoma were classified as malignancies. All of these lesions were defined as proliferative lesions in the glandular stomach. Gastric proliferative lesions were found only in groups 1-5. The incidence of total malignancies and

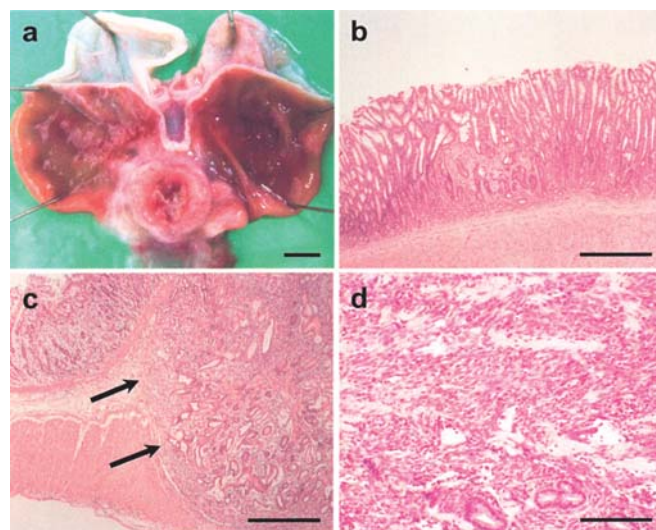


Figure 3. Macroscopic and microscopic appearance of MNNG-induced gastric tumors. (a) Representative macroscopic appearance of MNNG-induced tumor formation. Bar, 5 mm. (b-d) H&E staining of proliferative lesions in the stomach. (b) Atypical hyperplasia. Bar, 500 μm. (c) Invasive adenocarcinoma. Arrows indicate the invasion of cancer cells. Bar, 500 μm. (d) Spindle cell sarcoma. Bar, 100 μm.

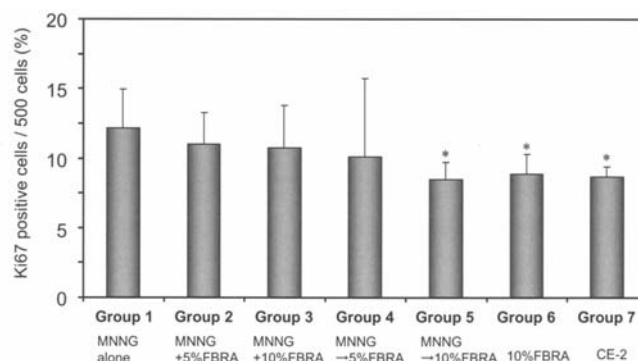


Figure 4. Effect of FBRA on the proliferation index in the non-lesional epithelium of the glandular stomach as detailed in Materials and methods. Columns, mean; Bars, SD; *P<0.05 versus group 1.

proliferative lesions in group 5 was significantly lower than that of group 1 (P<0.05). As shown in Table II, the mean number per animals (multiplicities) of total malignancies and proliferative lesions in group 5 was markedly decreased in comparison with that in group 1 (P<0.01). Furthermore, the number of atypical hyperplasias and invasive adenocarcinomas of group 5 were significantly smaller than that in group 1 (P<0.05). In addition, total number of malignancies in groups 4 and 5 were significantly smaller than that in group 1 (P<0.05).

Cell proliferation and apoptosis in the non-lesional gastric epithelium. The results of Ki67-labeling index in the non-lesional gastric epithelium of rats in each group are shown in Fig. 4. The Ki67-labeling index of group 5 was significantly smaller than that of group 1 (P<0.05). Similarly, the index of group 6 or 7 was smaller than that of group 1. Regarding apoptotic index, there were no statistically significant differences among the groups (data not shown).

Table I. The incidence rate of glandular stomach proliferative lesions in rats.

Group	Treatment	Effective no. of rats	AH ^a	No. of rats with proliferative lesions (%)					
				Malignancies					
				ADC ^b			Spindle cell sarcoma	Total	Total proliferative lesions
				Non-invasive ADC	Invasive ADC	Total			
1	MNNG	18	7 (39)	3 (17)	6 (33)	7 (39)	3 (17)	10 (56)	11 (61)
2	MNNG + 5% FBRA	21	7 (33)	2 (10)	4 (19)	5 (24)	1 (5)	5 (24)	8 (38)
3	MNNG + 10% FBRA	20	5 (25)	1 (5)	3 (15)	3 (15)	0	3 (15)	7 (35)
4	MNNG → 5% FBRA	20	3 (15)	1 (5)	3 (15)	4 (20)	1 (5)	5 (25)	6 (30)
5	MNNG → 10% FBRA	20	2 (10)	1 (5)	3 (15)	4 (20)	0	4 (20) ^c	5 (25) ^c
6	10% FBRA alone	20	0	0	0	0	0	0	0
7	Control vehicle	20	0	0	0	0	0	0	0

^aAH, atypical hyperplasia; ^bADC, adenocarcinoma. ^cP<0.05 versus group 1.

Table II. Multiplicities of glandular stomach proliferative lesions in rats.

Group	Treatment	Effective no. of rats	AH ^a	No. of gastric lesions per rat (mean ± SD)					
				Malignancies					
				ADC ^b			Spindle cell sarcoma	Total	Total proliferative lesions
				Non-invasive ADC	Invasive ADC	Total			
1	MNNG	18	0.56±0.78	0.17±0.38	0.78±1.22	0.94±1.30	0.17±0.38	1.67±1.23	1.67±1.57
2	MNNG+ 5%FBRA	21	0.57±0.98	0.14±0.4	0.33±0.80	0.48±1.17	0.05±0.22	0.52±1.21	1.10±1.70
3	MNNG+ 10%FBRA	20	0.75±1.45	0.05±0.22	0.20±0.52	0.25±0.64	0	0.25±0.64	1.00±1.65
4	MNNG → 5%FBRA	20	0.55±1.19	0.05±0.22	0.20±0.52	0.25±0.55 ^c	0.05±0.22	0.30±0.57 ^c	0.85±1.57
5	MNNG → 10%FBRA	20	0.15±0.49 ^c	0.05±0.22	0.15±0.37 ^c	0.20±0.41 ^c	0	0.20±0.41 ^d	0.35±0.67 ^d
6	10% FBRA alone	20	0	0	0	0	0	0	0
7	Control vehicle	20	0	0	0	0	0	0	0

^aAH, atypical hyperplasia; ^bADC, adenocarcinoma. ^cP<0.05 and ^dP<0.01 versus group 1.

Discussion

This study was undertaken in order to evaluate the efficacy of FBRA on carcinogen-induced gastric tumorigenesis in rats. In this study, administration of 10% FBRA in the diet during the post-initiation phase inhibited MNNG-induced gastric carcinogenesis in the level of the total number of total proliferative lesions, including malignancies. Furthermore, the treatment of 10% FBRA in the post-initiation phase also reduced the development of stomach adenocarcinomas in a dose-dependent manner. This effect of FBRA on the gastric carcinogenesis is consistent with the results of our previous studies where FBRA suppressed the carcinogenesis of the colon, liver, esophagus or urinary bladder by its exposure during the post-initiation phase (9,13-15).

Although the exact underlying mechanism for the suppressive effect of FBRA on gastric carcinogenesis is not clear, multiple constituents of FBRA are suggested to be related to the modifying effect. Antioxidants in FBRA such as

ferulic acid or phytic acid may be one of the responsive agents. In fact, the presence of a high amount of ferulic acid in FBRA is confirmed (23). Previously, our group reported a protective effect of ferulic acid, a polyphenol on the tongue or colorectal carcinogenesis in rodents (10,24). In another experiment, we also proved the suppressive effect of chlorogenic acid, a related antioxidative polyphenol, on N-methyl-N-nitrosourea-induced gastric carcinogenesis in rats (25). It is also known that dihydroferulic acid or dihydro-sinaptic acid contained in an unpolished rice vinegar possesses a potent radical scavenging activity (26). It is possible that the effect of FBRA is mediated through the action of several trace elements, including selenium which is biotransformed to an organic form during the fermentation of brown rice and rice bran (27-29).

In general, control of cell proliferation in the target organs for carcinogenesis is regarded as one of most important modes of action of chemopreventive agents (30). In this study, dietary exposure of FBRA significantly reduced Ki67-labeling

indices in the non-lesional gastric epithelium. Thus, the inhibitory effect of FBRA against carcinogen-induced hyperproliferation of the cells in the gastric mucosa is suggested to relate to the suppressive effect on the gastric carcinogenesis. Unfortunately, in this experiment, no clear difference in the apoptotic indices was found among each group in the analysis of apoptosis for the non-lesional gastric epithelium. Nevertheless, it may still be possible that dietary exposure of FBRA over a long-term during the post-initiation phase excludes carcinogen-induced initiated cells in the gastric epithelium. Recently, Sakurai *et al* (23) reported that the inhibitory effect of FBRA against liver metastasis of cancer cells is caused by cytokine generating type 1 helper T cell-dominant immune state and activation of macrophages. Such an immunostimulatory effect generated by the exposure of FBRA may also be involved in the suppressive effect on the gastric carcinogenesis in rodents.

In conclusion, we demonstrated that the dietary administration of 10% FBRA during the post-initiation phase suppresses MNNG-induced tumor development in the stomach of rats. FBRA is considered to be a promising chemopreventive agent against the occurrence of human gastric cancers.

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