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Prevention by long-term fermented miso of induction of colonic aberrant crypt foci by azoxymethane in F344 rats

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Abstract. The present study was designed to investigate the effects of fermented miso in the diet on the induction of aberrant crypt foci (ACF) by azoxymethane (AOM) in male F344 rats. A total of 50 rats, 8 weeks of age, were divided into 5 groups and given weekly subcutaneous injections of AOM (15 mg/kg body wt) for 3 weeks. Rats were fed a normal control MF solid diet, or solid diet containing 10% long-term fermented (aged), medium- or short-term fermented miso, or 2.2% NaCl for 5 weeks, starting one week before the first AOM dosing. It was found that, compared to the control (MF) diet, the long-term fermented diet significantly decreased (by 22.2%) ACF/colon, but increased (by 18.2%) the number of aberrant crypts (Acs)/focus. The latter was also increased by the medium-term fermented diet (by 25.3%). The PCNA labeling index was only affected by the short-term fermented diet (36.9% increase) and by 2.2% NaCl diet (27.2% increased). The present results indicate that aged or completely fermented miso supplemented into the diet, could act as a chemopreventive agent for colon carcinogenesis.

Introduction

Colorectal cancer is the second most frequent cause of cancer mortality in the USA and the third most common cancer worldwide. Recently, the progressive introduction of Western dietary habits has been paralleled by an increase in colon and breast cancer in Japan (1). Miso is fermented from soybeans, rice, wheat, or oats, and its major constituents are vitamins, microorganisms, salts, minerals, plant proteins, carbohydrates, and fat. It has traditionally been used in the daily diet as a flavor for food in Japan and other parts of Asia and is still one of the essential ingredients for Japanese-style cooking. We have described chemopreventive effects of miso against intestinal injury by X-irradiated mice (2) and it has also been reported to reduce the occurrence of liver (3,4) and gastric tumors (5) and aberrant crypt foci (ACF) of the colon in experimental animals (6). ACF are putative preneoplastic lesions of colon cancer that are utilized currently as biological end-points to evaluate modulation of colonic carcinogenesis. Masaoka *et al* earlier found dietary miso to inhibit the development of ACF in a dose-dependent manner (6). However, the effects of soy beans and the related food intake on cancer risk are complex (7). To determine whether soy beans themselves or fermentation processes may have a role, the present study was performed to assess the chemopreventive potential of samples after different periods of fermentation or aging.

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Materials and methods

Animals. Male F344/DuCrj rats, 6 weeks of age at the commencement, were purchased from Charles River and housed five to a polycarbonate cage under constant conditions of temperature (24±2˚C) and relative humidity (55±10%), with a 12:12-h light-dark cycle. The animals were maintained according to the 'Guide for the Care and Use of Laboratory Animals' established by Hiroshima University. All rats were fed a commercial diet (MF; Oriental Yeast Co., Tokyo, Japan) alone or with added miso and NaCl. Dry red miso after short- (immediate of fermentation), medium- (4 months of fermentation) and long-term fermentation (6 months) was supplemented into solid biscuits at 10% (Miso Central Institute, Tokyo, Japan). Similarly, NaCl was supplemented into biscuits at 2.2% (special grade, Wako Pure Chemical, Osaka, Japan), this concentration of corresponding to the concentration of salt in pure 10% miso paste. Normal tap water was also provided *ad libitum*.

Carcinogen. Azoxymethane (AOM) was purchased from Sigma Chemical Co. (St. Louis, MO).

Experimental procedure. A total of 50 rats were divided into 5 groups. Starting at 7 weeks of age, they were given weekly subcutaneous injections of AOM (15 mg/kg body wt) for 3 weeks to induce ACF. The rats were fed on the test

Table I. Mean body and organ weights (g) for rats in each group.

Group	Body weight	Liver	Kidney	Testis	Adrenal	Spleen
Long-term fermented miso	224.6±14.2	9.49±0.74[a]	1.80±0.11[c]	2.75±0.11	0.043±0.004[c]	0.610±0.026
Medium-term fermented miso	222.5±9.3	10.21±0.80	1.93±0.13	2.78±0.10	0.049±0.008	0.610±0.036
Short-term fermented miso	225.6±8.5	9.47±1.01[a]	1.78±0.18[c]	2.82±0.07	0.044±0.007[b]	0.624±0.035
NaCl	231.1±7.5	10.48±1.31	2.03±0.15	2.79±0.08	0.054±0.009	0.638±0.029
MF	232.4±10.2	10.65±0.83	1.94±0.12	2.73±0.06	0.046±0.009	0.642±0.044

Values are mean ± SD. [a]Significantly different from the MF value ($P<0.05$). [b]Significantly different from the NaCl value ($P<0.05$). [c]Significantly different from the NaCl value ($P<0.01$).

diets for 5 weeks, starting one week before the first AOM dosing. The animals were sacrificed 2 weeks after the last AOM injection.

Experimental groups. All animals were injected with AOM. Those in groups 1-3 were fed diets containing 10% miso (long-, medium- and short-term fermented miso, respectively). The animals in groups 4 and 5 were received the 2.2% NaCl and MF diets, respectively.

Autopsy of animals. Autopsy was performed under ether anesthesia at which time the body and major organ weights were measured.

Visualization and histological examination. At the termination of the studies, the colon was removed, flushed with saline, slit open longitudinally from cecum to anus, placed on a paper towel and fixed in 10% buffered formalin for 24 h. Following the protocol cited by Magnuson *et al* (8), the fixed colons were stained with 0.5% methylene blue for 15-30 min. The stained colons were then placed on a glass slide with the luminal side up. By viewing the stained colons with a light microscope at a magnification of x20-30 times, the colons were assessed for the presence of ACF. The numbers of ACF per colon, of AC per colon, and of AC per focus were determined.

Immunohistochemistry. An anti-PCNA antibody (Dako Co., Kyoto Japan) was used in the avidine-biotin complex method. Tissue sections were deparaffinized with xylene, hydrated through a graded ethanol series and incubated with 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. They were then incubated with 10% normal house serum at room temperature for 30 min to block background staining and then with the anti-PCNA antibody. The locations of the nearest and furthest PCNA-positive cells from the bed of crypt were defined as the base and the top, and the distance between the germinal region (9). Cell numbers observed in single half crypts from the beds of crypts to the surface were counted and converted into % values for bottom and top positions. Numbers of positively stained nuclei were counted and divided by the total number of nuclei to give the PCNA index (%).

Statistics. Statistical significance was determined with Dunnett's method for multiple comparisons using logarithmic transformation and the Student's t-test.

Results

General observations. Intake of diet and drinking water did not differ among the groups (data not shown). Body and organ weights are shown in Table I. Average body weights of rats were not significantly different among groups. Liver weights (absolute and relative weights) in short- and long-term fermented miso groups were significantly decreased as compared with values of the MF group. Kidney and adrenal weights (absolute and relative weights) in the short- and long-term fermented miso groups were significantly decreased as compared with values of the NaCl group, but values were all within the biological ranges (Table II).

Colonic ACF. Table III summarizes data for the mean numbers of ACF per colon, total number of AC per colon and mean number of AC per focus. The rats were all treated with AOM and showed a 100% incidence. The average number of ACF per colon was significantly decreased in the long-term fermented miso group as compared with the short-, medium-term fermented miso and MF ($P<0.05$). ACs per colon were significantly greater in the medium-term fermented miso compared to the long-term fermented miso cases ($P<0.05$). The NaCl group demonstrated no statistically significant differences from the MF group. Long-term fermented and medium-term fermented miso were associated with significant increase in the numbers of AC per focus as compared to the MF group ($P<0.01$).

PCNA. The results for PCNA-positive indices in the colonic mucosal epithelium are shown in Table IV. The number of cells in each half crypt with medium-term fermented was significantly increased as compared with the long-term fermented miso or MF values but without any clear relation

Table II. Relative organ weights.

Group	Liver	Kidney	Testis	Adrenal	Spleen
Long-term fermented miso	42.51±5.15	8.05±0.90[b]	12.29±0.70	0.19±0.03[b]	2.73±0.21
Medium-term fermented miso	45.91±3.17	8.67±0.49	12.49±0.49[a]	0.22±0.03	2.74±0.11
Short-term fermented miso	41.94±3.55	7.87±0.68[b]	12.50±0.42[a]	0.19±0.03[b]	2.77±0.19
NaCl	45.25±4.45	8.80±0.47	12.08±0.54	0.23±0.03	2.76±0.17
MF	45.83±2.87	8.37±0.43	11.77±0.49	0.20±0.03	2.76±0.10

Values are mean ± SD. [a]Significantly different from the MF value (P<0.05). [b]Significantly different from the NaCl value (P<0.05).

Table III. Effects of dietary miso on AOM induction of ACFs in rat colon.

Group	Number of rats	Incidence	ACF/colon	ACs/colon	ACs/Focus
Long-term fermented miso	10	10	65.1±18.4	138.2±37.1	2.01±0.13[b]
Medium-term fermented miso	10	10	90.9±30.9[a]	200.3±73.6[a]	2.13±0.23[b]
Short-term fermented miso	10	10	85.5±19.4[a]	158.2±27.9	1.85±0.13
NaCl	7	7	84.9±52.1	143.7±83.0	1.73±0.22
MF	13	13	83.7±21.3[a]	147.7±58.4	1.70±0.24

Values are means ± SD; ACF, aberrant crypt foci; ACs, aberrant crypt. [a]Significantly different from the long-term fermented group value (P<0.05). [b]Significantly different from the MF group value (P<0.01).

Table IV. Number of cells, labeling index, and germinal region.

Group	No. of cells in half crypt	Bottom	Top	Germinal region width	Labeling index
Long-term fermented miso	32.3±2.9	12.8±10.7[a]	52.7±11.6	39.9±14.4	22.1±7.3
Medium-term fermented miso	34.7±2.7[b,c]	10.0±8.2[d]	50.6±11.2[a]	40.6±12.8	23.3±8.3
Short-term fermented miso	32.0±3.6	9.6±8.8[d]	57.1±11.6[d]	47.5±15.0[b,c]	29.7±8.9[b,c]
NaCl	31.6±2.7	9.5±8.3[d]	51.3±12.1	41.8±13.6	27.6±9.6[b,c]
MF	32.2±2.9	12.6±10.1	54.8±12.4	42.2±14.8	21.7±6.9

[a]Significantly different from the MF group (P<0.05). [b]Significantly different from the MF group (P<0.01). [c]Significantly different from the long-term fermented miso group (P<0.01). [d]Significantly different from the long-term fermented miso group (P<0.05).

to the PCNA indices. The cells located at the bottom in the long-term fermented miso group were significantly higher than in the medium-term fermented, short-term fermented miso or NaCl cases. The cells located at top with short-term fermented were significantly higher than with long-term fermented miso and with medium-term fermented miso lower than the MF value.

Discussion

In the present study, dietary administration of long-term fermented miso inhibited the development of AOM-induced ACF in the rat colon. It has been concluded that consumption of soy foods may contribute to the relatively low rates of breast, colon, and prostate cancers in countries such as China and Japan (10). Tuyns *et al* reported soybean to be clearly protective against colon rectal cancers on the basis of a case control study in Belgium (11). Soy foods contain significant amounts of two isoflavones, genistein and daidzein (12), which have various biological activities and antitumorigenic effects (7). Fukutake and colleagues (13) reported that genistein is present at higher levels in miso than in other soybean-related products, such as soy powder, soy milk, tofu, natto and soy sauce. This has been proposed as the most effective constituent for inhibition of mammary cancer, also possessing antiestrogenic activity (14-17). Protection against mammary and prostate tumors is also expected with isoflavones, but one group found no difference in DMH-induced colon cancers when comparing diets containing soy protein (18). However, Thiagrajan *et al* observed a protective effect of soy products against induction of ACF (19). They and Pereira *et al* (20) also found purified genistein to inhibit induction of ACF, although Rao *et al* reported this agent to increase tumor numbers in rat colon after treatment with AOM (21). Azuma *et al* reported the antitumorigenicity of the high-molecular-weight fraction after digestion of soy protein isolate with protease to be due to the inhibitory effect of soybean resistant proteins (22-24). Gotoh and associates (25) demonstrated that administration of biochanin A, a genistein precursor, inhibits the development of rat mammary tumors. However, we previously found that it did not reduce colonic ACF in rats induced by AOM (26). Thus inhibitory effects of miso against colorectal and mammary cancers may be due to different constituents. Kawamori and others (27) reported that a saponin in soybean inhibited ACF induction by AOM in rats. Recently we found that prolonged fermentation might be very important for protection against radiation effects, being associated with prolongation of animal survival and decrease in toxicity to small intestinal crypts (29). However, to our knowledge there are no reports regarding the effective substances in miso after different periods of fermentation. Further study is now ongoing for the long-term experiment.

In the present study, long-term fermentation appeared linked to significant decrease in the PCNA-positive index as compared to short-term fermented miso and NaCl groups. The results are in line with an earlier report that BrdU labeling index with 10% miso supplementation was significantly lower than that of normal diet group in AOM treated rats and also the length of germinal region (6) and PCNA positive index were reduced by a water soluble extract from cultured medium of *Ganoderma lucidum* (Rei-Shi) mycelia (9). Calcium has been hypothesized as a regulator of cell proliferation in the colon, and dietary intake of calcium inhibits experimental carcinogenesis (28). Its presence in miso could therefore be important. Which other components might be involved and specifically those which increase with the period of fermentation, remains to be determined.

In conclusion, the results of the present study indicate that fermented miso supplementation of the diet might be useful for colonic cancer prevention. Further experiments are required to evaluate the effects of individual components, including minerals, in miso.

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