

Protective Effects of Japanese Black Vinegar “*Kurozu*” and Its Sediment “*Kurozu Moromimatsu*” on Dextran Sulfate Sodium-induced Experimental Colitis

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1. Introduction

Kurozu is a traditional Japanese black vinegar that is used in the preparation of foods. It is manufactured, mainly in Kagoshima prefecture in Japan, by fermentation of unpolished rice with lactobacillus and *Koji* bacillus in earthenware jars for more than one year, during which time it gradually becomes black. The supernatant is known as *Kurozu*, and the solid sediment, which is rich in organic materials, minerals, amino acids and so on, is known as *Kurozu Moromimatsu* (*Kurozu-M*). Many products containing *Kurozu* and *Kurozu-M* are available in Japan as health foods or supplements.

As reported by Murooka et al. (Murooka Y & Yamashita M, 2008), *Kurozu* has ameliorating effects on hyperlipemia and hypertension, as well as anti-cancer activity against colon cancer in vitro and in vivo (Nanda K et al., 2004; Shimoji Y et al., 2003; Shimoji Y et al., 2004). Further, we reported that *Kurozu-M* treatment reduced the activity of gelatinases (metalloproteinase-2, -9) in tumor tissues, inhibited the growth of human colon cancer cells, LoVo, in an animal model (Fukuyama N et al., 2007), and inhibited the growth of hepatocellular carcinoma in a diethylnitrosamine-induced animal model (Shizuma T et al., 2011). *Kurozu* has also been reported to have free radical-scavenging activity (Murooka Y & Yamashita M, 2008). Since active oxygen species or radicals are related to inflammation and tissue injury, *Kurozu* and *Kurozu-M* may be potential functional foods with preventive or therapeutic effects against inflammatory diseases.

Ulcerative colitis (UC) is an obstinate inflammatory bowel disease (IBD). The causes of UC are not well-established, but multiple genetic factors (van Lierop et al., 2009), immune responses of the colon (Hong SK et al., 2010) intestinal flora, and inflammatory cytokines (Polińska B et al., 2009; Ghosh N et al., 2010) have been suggested to be involved. Moreover, oxidative stress is thought to influence the severity of UC (Beckman JS et al., 1990; Babbs CF, 1992; Grudziński IP & Frankiewicz-Józko A, 2001; Hong SK et al., 2010). Enhanced release of reactive oxygen species (ROS), such as superoxide and hydroxyl radical, and reactive nitrogen species (RNS), such as peroxynitrite generated from nitric oxide (NO), is associated with aggravation of both clinical UC and dextran sulfate sodium (DSS)-induced colitis in an animal model (Elson CO et al., 1995).

Therefore, we decided to examine the protective effects of *Kurozu* and *Kurozu-M* in a rodent model of DSS-induced colitis, focusing on the possible role of anti-oxidative and anti-nitration effects.

2. Main body

2.1 Materials and methods

The experimental procedures were approved by the Animal Experimentation Committee, School of Medicine, Tokai University, Japan.

2.1.1 Experimental model

A solution of 3.5% DSS was given orally for 12 days to forty C57black6 female mice (CLEA Japan Inc., Tokyo, Japan). The mice were divided into 4 groups: the control group received the standard CE-2 rodent diet (CLEA Japan Inc., Tokyo, Japan) (n=10), the *Kurozu* group received CE-2 diet containing 3.2% solution of *Kurozu* (n=10), the *Kurozu-M* group received CE-2 diet including 2% *Kurozu-M* (n=10), and the acetic acid group received CE-2 diet including 0.3% solution of acetic acid (n=10). The amounts of *Kurozu*, *Kurozu-M* and acetic acid were based on volumes typically ingested by humans, adjusted for body weight. All mice were bred under specific pathogen-free conditions, because clinical UC is influenced by intestinal flora. CE-2 is a standard rodent diet, and includes soybean or white fish meal as source of protein, soybean oil or germ as source of lipids, rice bran or alfalfa as a source of carbohydrate, vegetable fiber, several vitamins, and minerals.

The three rodent diets other than the standard CE-2 diet were supplied by Sakamoto *Kurozu* Inc. (Kagoshima, Japan). The standard CE-2 and the three special diets were started a week before the initiation of oral DSS administration.

2.1.2 Experimental procedures

After initial DSS administration, changes of body weight and bloody stool frequency were monitored every 2 days for 12 days in all mice. Then, the mice were sacrificed and the proximal colon was resected. Microscopic examination (hematoxylin-eosin (H.E.) staining) was performed in all groups, and myeloperoxidase (MPO) staining, as a marker of leukocyte activation, was performed for all groups except the acetic acid group. Moreover, histological findings in the colon were evaluated and scored in the 4 groups according to the reported grading system (Tomita T et al., 2008), as follows. Mucosal damage: 0, normal; 1, 3-10 intraepithelial cells (IEL)/high power field (HPF) and focal damage; 2, >10 IEL/HPF and rare crypt abscesses; 3, >10 IEL/HPF, multiple crypt abscesses and erosion/ulceration. Submucosal damage: 0, normal or widely scattered leukocytes; 1, focal aggregates of leukocytes; 2, diffuse leukocyte infiltration with expansion of submucosa; 3, diffuse leukocyte infiltration. Muscularis damage: 0, normal or widely scattered leukocytes; 1, widely scattered leukocyte aggregates between muscle layers; 2, leukocyte infiltration with focal effacement of the muscularis; 3, extensive leukocyte infiltration with transmural effacement of the muscularis.

Moreover, enzyme-linked immunosorbent assay (ELISA) of serum tumor necrosis factor (TNF)- α and interleukin (IL)-2 as pro-inflammatory cytokines was carried out in the control

and *Kurozu* groups. ELISA measurement of nitrotyrosine levels of resected colonic tissues at 12 days after initial DSS administration, as a marker of oxidative or nitration stress, was performed in all groups except the acetic acid group. Moreover, urinary excretion/day (during 11-12 days after initial DSS administration) of nitrite and nitrate (NO_x) as a parameter of the bioavailability of NO was measured by means of the Griess method (Griess reagent kit; Invitrogen Japan K.K, Tokyo, Japan) in the (DSS-induced) control group, the *Kurozu* group, and the group given standard CE-2 diet without administration of DSS (each group: n=10).

2.1.3 Statistical analysis

The significance of differences of body weight, serum cytokines (TNF- α and IL-2), histological scores, and nitrotyrosine and NO_x levels among the groups was examined by one-way analysis of variance (ANOVA) and Tukey’s multiple comparison post-hoc test. The significance of differences in bloody stool frequency was examined by contingency table analysis. The criterion of significance was $p < 0.05$.

Body weight after DSS administration is given as a percentage of basal body weight before DSS administration, taken as 100%. Levels of serum cytokines (TNF- α and IL-2) and histological scores, nitrotyrosine in colonic tissues, and NO_x in urine, are presented as mean and standard deviation (SD). The frequency of mice with bloody stool after DSS administration is given as a percentage of the number of animals in each group.

2.2 Results

2.2.1 Change of body weight

There were no significant differences of diet or water intake among the four groups throughout the 12 days (data not shown).

The *Kurozu* group showed a significantly reduced body weight loss in the period of 6-12 days after initial DSS administration compared with the control group ($p < 0.001$) and in the period of 8-12 days after initial DSS administration compared with the acetic acid group ($p < 0.001$). The *Kurozu-M* group showed a significantly reduced body weight loss in the period of 6-8 days after initial DSS administration compared with the control group ($p < 0.05$) and at 8 and 12 days after initial DSS administration compared with the acetic acid group ($p < 0.05$). The acetic acid group showed a significantly reduced body weight loss only at 6 days after initial DSS administration compared with the control group ($p < 0.01$). The results are summarized in Table 1.

2.2.2 Frequency of bloody stool

The appearance of bloody stool was noted in all mice of the control and acetic acid groups in the period of 4-12 days after initial DSS administration. In contrast, bloody stool was rarely noted in the *Kurozu* group: the frequency was 0% (0/10) during 2-8 days after initial DSS administration and only 20% (2/10) at 12 days after initial DSS administration. Similarly, in the *Kurozu-M* group, bloody stool was not noted during 2-8 days after initial DSS administration and the frequency was only 30% (3/10) at 12 days (Table 2).

	2	4	6	8	10	12 (days) [*]
control group	97.1 ±1.2	95.9 ±3.3	90.6 ±3.3	80.8 ±2.2	73.4 ±2.1	71.1 ±1.6
<i>Kurozu</i> group	99.2 ±2.2	98.2 ±1.8	97.8 ±1.3	89.4 ±2.9	87.2 ±2.9	81.6 ±2.4
<i>Kurozu-M</i> group	97.6 ±3.1	97.2 ±1.9	96.2 ±3.6	86.1 ±3.6	76.8 ±2.9	74.4 ±3.9
acetic acid group	99.2 ±2.3	97.8 ±1.9	96.6 ±2.1	80.1 ±2.3	72.0 ±3.5	69.1 ±3.1

^a p<0.001; ^b p<0.01; ^c p<0.05; *days after initial administration of DSS

Body weight after DSS administration is given as a percentage of the basal body weight before DSS administration, taken as 100% (mean ±SD). (Shizuma T et al., 2011)

Table 1. Changes of body weight after initial administration of DSS

	2	4	6	8	10	12 (days) [†]
control group	9/10 (90%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)
<i>Kurozu</i> group	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	1/10 (10%)	2/10 (20%)
<i>Kurozu-M</i> group	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	2/10 (20%)	3/10 (30%)
acetic acid group	7/10 (70%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)

*p<0.01; †days after initial administration of DSS

The bloody stool frequency in mice after initial DSS administration is given as a percentage of the number of animals in each group. (Shizuma T et al., 2011)

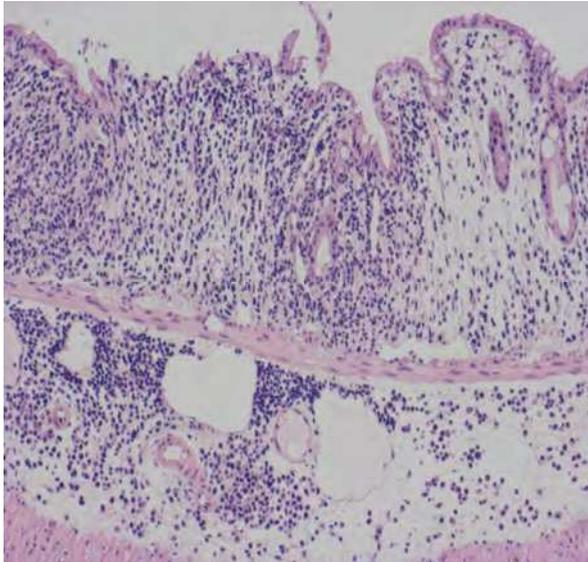
Table 2. Frequencies of bloody stool after initial administration of DSS

2.2.3 Histology

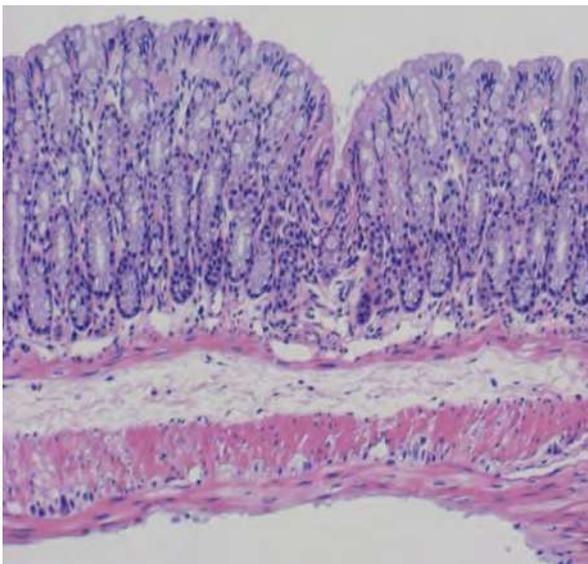
H.E. staining revealed epithelial abrasions, cryptal disturbance, hemorrhage and inflammatory cell invasion, and thickening of layers in the mucosa and submucosa of the colon in the control group, but these changes were minimal in the *Kurozu* and *Kurozu-M* groups. There was no marked difference between the control group and the acetic acid group (Fig.1a~d). Moreover, the *Kurozu* and the *Kurozu-M* groups showed fewer MPO-positive cells than the control group (Fig. 2a~c).

Histological scores (Tomita T et al., 2008) were as follows: control group, 5.88±0.33; *Kurozu* group, 0.50±0.50; *Kurozu-M* group, 0.88±0.33; acetic acid group, 4.38±0.48. In the *Kurozu*

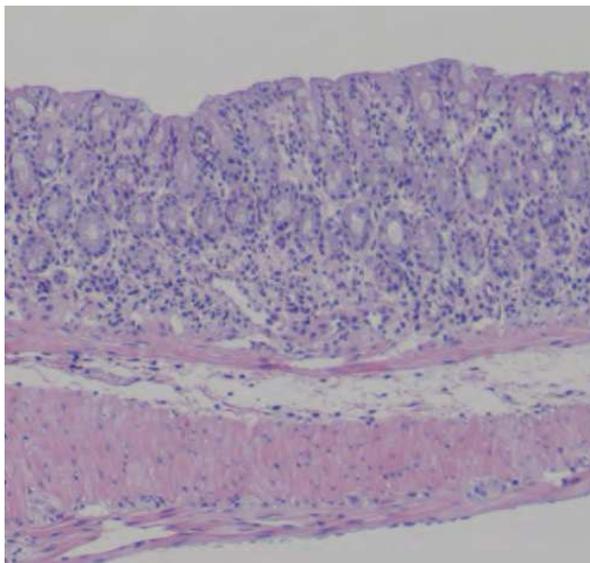
group and *Kurozu-M* groups, the scores were significantly ($p < 0.001$) reduced compared with the control and acetic acid groups. Moreover, there was no significant difference between the control group and acetic acid group.



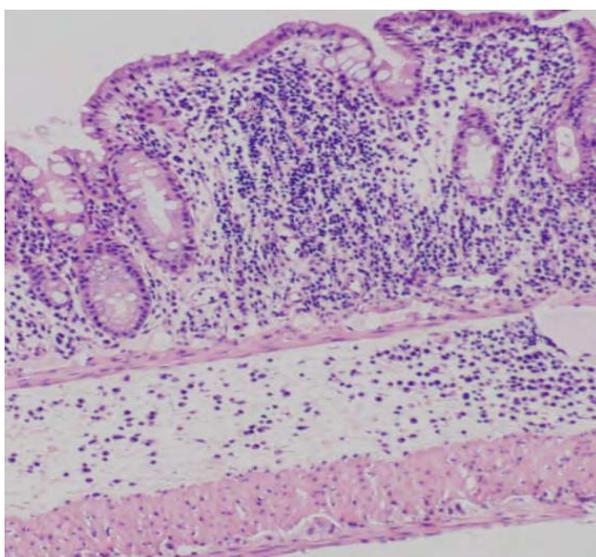
(a) control group



(b) *Kurozu* group



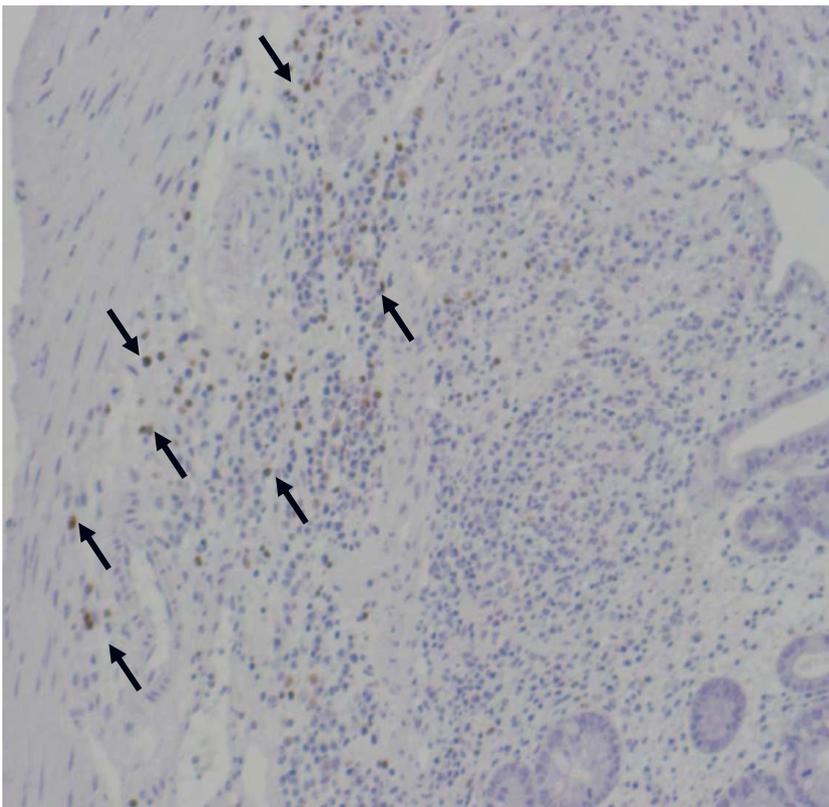
(c) *Kurozu-M* group



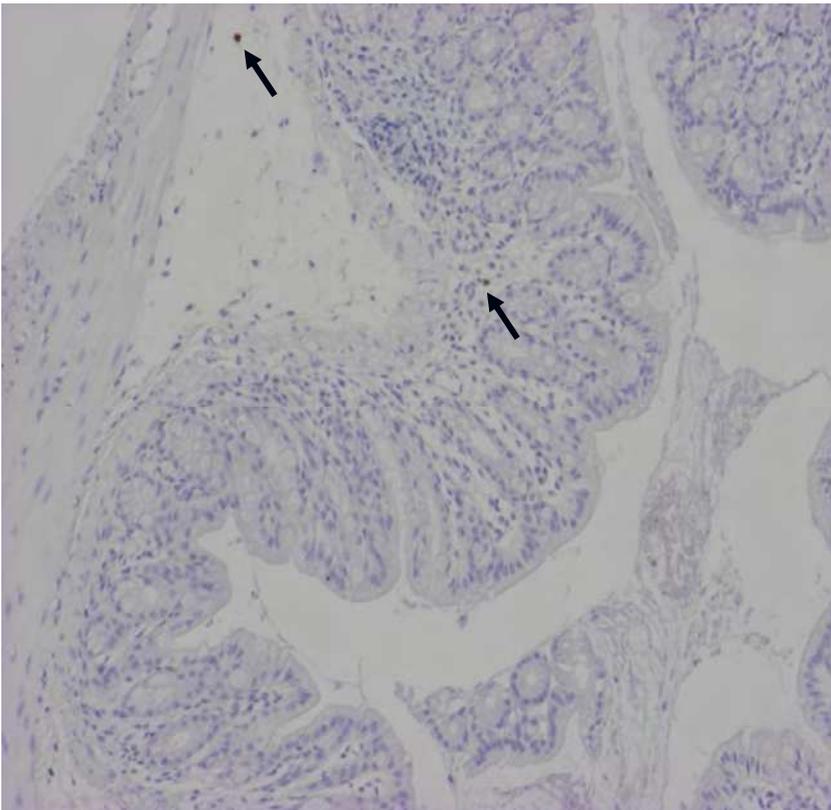
(d) acetic acid group

H.E. staining of resected colon revealed abrasions of epithelium, cryptal disturbance, and inflammatory cell infiltration in mucosa and submucosal areas of colon in the control group. *Kurozu* and *Kurozu-M* treatment remarkably attenuated these changes in comparison to the control group. The acetic acid group showed no marked attenuation of colitis compared with the control group (a, the control group; b, the *Kurozu* group; c, the *Kurozu-M* group; d, the acetic acid group).

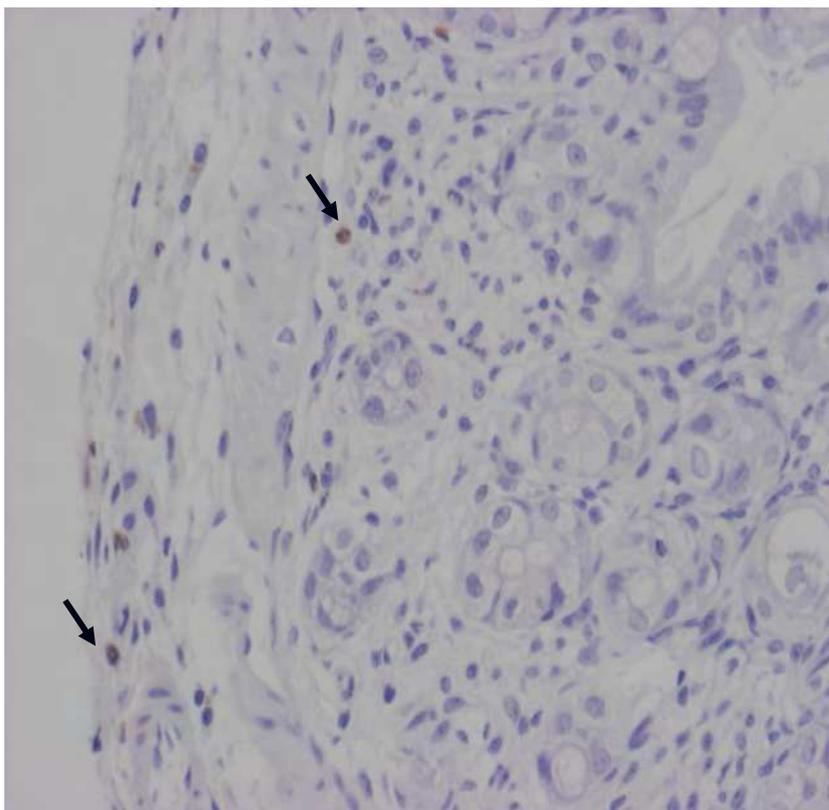
Fig. 1 Histological findings of resected colon



(a) Control group



(b) *Kurozu* group



(c) *Kurozu-M* group

MPO-positive cells are indicated by ↗ and ↘ (a, control group; b, *Kurozu* group; c, *Kurozu-M* group). The *Kurozu* and the *Kurozu-M* groups showed fewer MPO-positive cells than the control group.

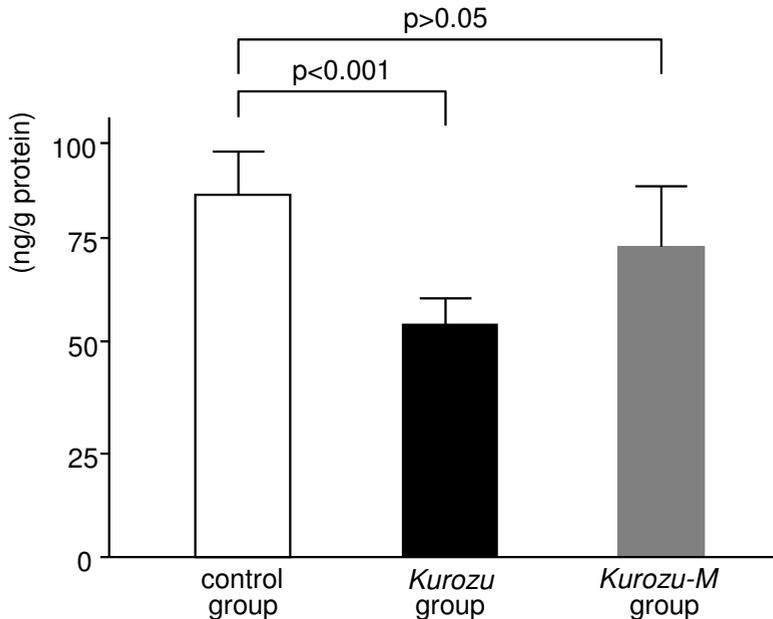
Fig. 2. MPO staining in colonic tissue

2.2.4 Levels of cytokines

The levels of TNF- α (pg/ml) were 17.2 ± 3.25 in the control group with administration of DSS and 15.9 ± 5.77 in the *Kurozu* group (normal range: 0.6~2.8). Levels of IL-2 (U/ml) were 1.23 ± 0.37 in the control group with administration of DSS and 1.13 ± 0.39 in the *Kurozu* group (normal range: below 0.8). There were no significant differences in the levels of either of the cytokines between the two groups.

2.2.5 Level of nitrotyrosine in colonic tissue

The *Kurozu* group showed a significantly reduced nitrotyrosine level (53.1 ± 7.1 ng/g protein) in resected colonic tissue compared with the control group (86.9 ± 11.7 ng/g protein, $p < 0.001$). However, the difference from the control was not significant in the *Kurozu-M* group (74.2 ± 15.1 ng/g protein) (Fig. 3).

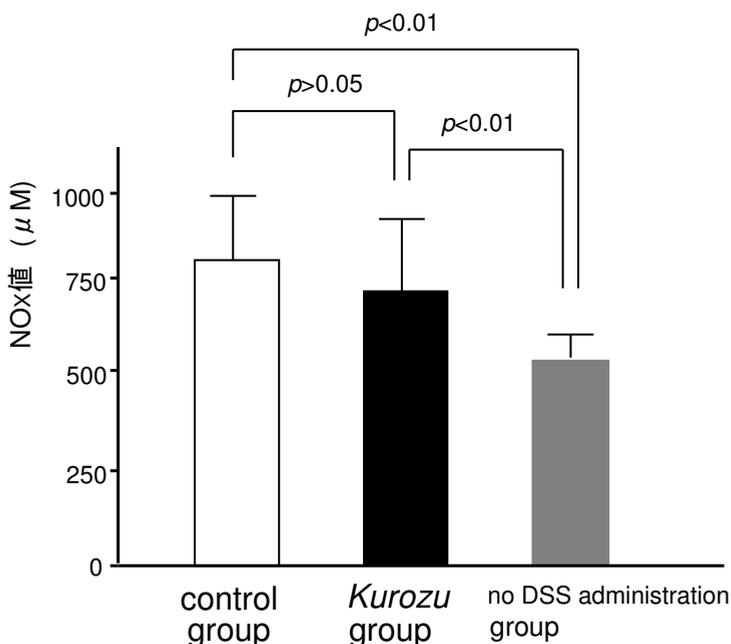


Kurozu treatment significantly ($p < 0.001$) reduced the nitrotyrosine level in resected colonic tissue, in comparison to the control group. On the other hand, the difference from the control was not significant in the *Kurozu-M* group.

Fig. 3. Nitrotyrosine levels in resected colonic tissue

2.2.6 Level of NOx in urine

NOx levels in urine (μM) in the (DSS-induced) control group (807 ± 172) and the *Kurozu* group (723 ± 196) were significantly increased compared with the group that did not receive DSS administration (517 ± 87). However, there were no significant difference between the (DSS-induced) control group and the *Kurozu* group (Fig. 4).



NOx levels in urine (μM) in the (DSS-induced) control group and the *Kurozu* group were significantly increased compared with the control group without DSS administration. However, there was no significant difference between the (DSS-induced) control group and the *Kurozu* group.

Fig. 4. NOx levels among the three groups

2.3 Discussion

Our results indicate that *Kurozu* and *Kurozu-M* exert protective effects against DSS-induced colitis in mice. *Kurozu* and *Kurozu-M* both reduced the bloody stool frequency and attenuated inflammatory changes of colon tissue, as well as inhibiting body weight loss.

Further, *Kurozu* and *Kurozu-M* decreased the number of MPO-positive cells. Since MPO is a marker of activation of leukocytes (Schindhelm RK., 2009), both *Kurozu* and *Kurozu-M* treatments appear to have anti-inflammatory effects in our model. Moreover, *Kurozu*, but not *Kurozu-M*, significantly reduced the nitrotyrosine level in colonic tissues in comparison with the control, although there was no significant difference in NOx level between the control and *Kurozu* groups. Nitrotyrosine is produced via at least two pathways, reaction of superoxide and NO, and reaction of nitrite and MPO (Beckman JS et al., 1990; Radi R, 2009). Therefore, *Kurozu* may suppress either of these pathways, or both. On the other hand, NOx is a good index of generation of NO by NO synthase (NOS) (Akuta T et al., 2006; Yang GY et al., 2009). Since no significant reduction of NOx level by *Kurozu* treatment was found in this study, suppression of NO and nitrite formation can be ruled out as a mechanism of reduction of nitrotyrosine generation. Therefore, *Kurozu* may reduce nitrotyrosine formation by suppressing the generation of superoxide or the activity of MPO.

In inflammatory states, including UC, generation of superoxide and NO is generally accelerated (Cross RK & Wilson KT, 2003). Superoxide itself is cytotoxic and is associated with tissue damage in many diseases. Moreover, superoxide and NO rapidly react with each other to form highly reactive peroxynitrite, leading to severe tissue damage via nitration of protein tyrosine residues to form nitrotyrosine, which is consequently considered to be a marker of oxidative and nitration stress. Therefore, peroxynitrite may have played a key role in the induction of colitis in this study.

Regarding the MPO pathway, MPO is mainly released from activated neutrophils. Since it is possible that *Kurozu* may block MPO release from neutrophils in the colonic tissues, we can not rule out the possibility that *Kurozu* suppresses the reaction of MPO and nitrite, thereby leading to a reduction of nitrotyrosine formation. However, *Kurozu-M* treatment did not reduce the generation of nitrotyrosine significantly, although infiltration of MPO-positive cells was remarkably suppressed. Moreover, the cytotoxicity of MPO itself is unclear; our previous study demonstrated that MPO was protective against tissue injury in MPO knock-out mice, although in that case, we examined brain tissue (Takizawa S et al., 2002). However, at present, we have no evidence that the reduction of nitrotyrosine level in the *Kurozu* group involves suppression of MPO. Rather, our findings indicate that the mechanism predominantly mediating the anti-colitis effect of *Kurozu* is suppression of superoxide production, leading to decreased generation of peroxynitrite, which is strongly cytotoxic, or other ROS and RNS, although at this stage we can not rule out the possibility that suppression of MPO also contributes.

Overall, our results indicate that *Kurozu* and *Kurozu-M* both have an anti-colitis effect in the DSS-induced mouse colitis model, though *Kurozu* is more potent. This, in turn, suggests that the active materials are predominantly soluble compounds, because *Kurozu-M* is the precipitate formed during the manufacture of *Kurozu*.

Kurozu contains acetic acid, free amino acids, peptides, minerals, water-soluble vitamins, organic compounds including bacterial fermentation products, lipids and saccharides. Its main component is acetic acid, but we found here that acetic acid did not affect body weight loss, bloody stool frequency, or pathological findings in DSS-induced mice, compared with the control. Further, acetic acid is not present in *Kurozu-M*. Therefore, acetic acid is not one of the major protective components against colitis.

Amino acids seem to be good candidates for the anti-colitis agents in *Kurozu* and *Kurozu-M*. For example, glutamine has a protective effect on gut function, and has been suggested to have an anti-colitis effect in an animal model (Ameho CK et al., 1997). Other amino acids or their metabolites or degradation products, including arginine, asparagine, cysteine, serine (Faure M et al., 2006), methionine, and tryptophan, are known to have anti-oxidative effects (Wu G, 2009). Moreover, cysteine (Kim CJ et al., 2009), serine and tryptophan (Kim CJ et al., 2010) were found to have protective effects in animal models of colitis. Therefore, free amino acids or peptides may be among the active agents present in *Kurozu* and *Kurozu-M*.

Other organic materials, including lactic acid and products of lactobacillus or *Koji* bacillus fermentation, are also candidate protective agents. *Kurozu* is fermented with lactobacillus or *Koji* bacillus for several years in earthenware jars, and lactobacillus is well known as a beneficial component of human intestinal flora. Moreover, clinically significant anti-colitis effects of administration of probiotics (Sartor RB, 2004) or synbiotics (Kanauchi O et al.,

2009) have been reported in patients with mild UC. Further, anti-oxidative effects and protective effects against animal colitis were reported for *Koji* bacillus (Fukuda Y et al., 2006; Lee IH et al., 2008). Therefore, many components of *Kurozu* and *Kurozu-M*, including amino acids and oligopeptides, as well as other organic materials, may contribute to the anti-colitis effects observed here.

At present, UC is generally treated with anti-inflammatory agents, such as 5-aminosalicylic acid (5-ASA) and prednisolone (Rogler G, 2009). However, administration of these drugs is sometimes accompanied with severe side effects. Therefore, a dietary therapy would be desirable, particularly from the viewpoints of safety and long-term effectiveness.

In order to further examine the mechanism of action of *Kurozu*, additional study will be needed to evaluate the quantitative changes of pro-inflammatory cytokines in resected colonic tissues, for example, measurement of mRNA expression levels in tissues of the DSS-induced control and *Kurozu* groups, because there was no difference in the serum levels of pro-inflammatory cytokines. It could not be determined in this study whether *Kurozu* has a direct anti-oxidant effect, because the absorption, distribution, metabolism and excretion characteristics of *Kurozu* and its constituents in the mouse remain to be determined. This question might be addressed by means of in vitro experiments on colonic cell lines or rectal application of *Kurozu* in an animal model.

For the practical use of *Kurozu* in the management of colitis patients, it will be necessary to identify optimum dosages and administration schedules. Many products containing *Kurozu* are available in Japan as health foods or supplements, and in this study, the amounts of *Kurozu* used were based on volumes typically ingested by humans, adjusted for body weight. The next step will be a clinical trial to examine the anti-colitis effects of *Kurozu* as an oral supplement in patients with mild ulcerative colitis, focusing initially on typical dose and administration schedules recommended for existing health foods and supplements.

3. Conclusion

Our results indicate that *Kurozu* exerts a protective effect against DSS-induced colitis in mice, and one of the mechanisms involved may be an anti-oxidative or anti-nitration stress activity.

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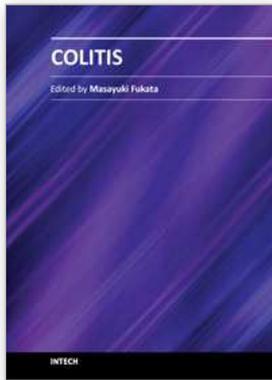
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Inflammation of the colon is collectively called "Colitis". Since a variety of conditions may cause colitis and its manifestations are similar among the causes, selection of the right treatment based on the correct diagnosis is important in the management of this group of illnesses. Over the last few decades, a major shift has been observed in the clinical attention to the pathogenesis of colitis from infectious to idiopathic inflammatory bowel diseases. Colitis cases that are associated with chemical therapeutics and specific pathogens such as amoeba, have become prominent in hospitalized individuals and immune deficient patients, respectively. In addition, a great deal of progress has been made in colitis research triggering the need for updating our knowledge about colitis. This book Colitis provides comprehensive information on the pathogenesis, mechanism of resolution, and treatment strategies of colitis.

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