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Anti-inflammatory effects of orally administered glucosamine oligomer in an experimental model of inflammatory bowel disease

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ABSTRACT

Anti-inflammatory effects of oral administration of the glucosamine oligomers (chito-oligosaccharides: COS) were evaluated in an experimental model of inflammatory bowel disease (IBD). Oral administration of COS improved shortening of colon length and tissue injury (as assessed by histology) in mice. Oral administration of COS inhibited inflammation in the colonic mucosa by suppression of myeloperoxidase activation in inflammatory cells, as well as activation of nuclear factor-kappa B, cyclooxygenase-2, and inducible nitric oxide synthase. Oral administration of COS also reduced serum levels of pro-inflammatory cytokines (tumor necrosis factor-α and interleukin-6). Moreover, it prolonged survival time in mice. These data suggest that COS have anti-inflammatory effects in an experimental model of IBD, and could be new functional foods for IBD patients.

1. Introduction

Inflammatory bowel disease (IBD) encompasses ulcerative colitis (UC) and Crohn's disease, and is characterized by chronic inflammation of the gut (Morrison, Headon, & Gibson, 2009). The incidence of IBD has increased steadily in some areas of the world over the past 40 years (Goh & Xiao, 2009), possibly because of changes in dietary habits (particularly consumption of diets low in fiber content) (Rose, DeMeo, Keshavarzian, & Hamaker, 2007). The goal of IBD treatment is to achieve and maintain remission (Pithadia & Jain, 2011). However, drugs not only have beneficial effects but also have adverse effects in IBD patients (Morrison et al., 2009).

Chitooligosaccharides (COS: glucosamine oligomers) have been reported to have anti-tumor (Harish-Prashanth & Tharanathan, 2005; Huang, Mendis, Rajapakse, & Kim, 2006; Shen, Chen, Chan, Jeng, & Wang, 2009; Suzuki et al., 1986; Tokoro, Tatewaki, Mikami, Suzuki, & Suzuki, 1989; Wang et al., 2008) and anti-inflammatory (Li, Liu, Xu, Du, & Xu, 2014; Wei et al., 2012) activities. Moreover, Yousef, Pichyangkura, Soodvilai, Chatsudthipong, and Muanprasat (2012) demonstrated that intraperitoneal injection of COS suppressed inflammation of the colon in an experimental model of IBD (Yousef et al., 2012). However, most reports that describe the bioactivities COS have evaluated intraperitoneal or intravenous administration of COS *in vivo* or *in vitro*.

Recently, it was revealed that oral administration of chitin nanofibrils or glucosamine from chitin led to anti-inflammatory effects in experimental models of IBD (Azuma et al., 2012a; Azuma et al., 2012b; Yomogida et al., 2008). These results indicate chitin derivatives have a potency as an anti-inflammatory agents. However, reports describing the anti-inflammatory effects of COS by oral administration in an experimental model of IBD are lacking; hence, the present study was undertaken.

2. Materials and methods

2.1. Reagents

COS, derived from crab shells, was provided by Koyo Chemical Co. Ltd. (Tokyo, Japan). COS comprise dimers (8.9%), trimers (19.6%),

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tetramers (23.7%), pentamers (25.4%), hexamers (8.4%), heptamers (3.1%), and octamers (1.7%) (Masuda et al., 2014). Dextran sulfate sodium (DSS; molecular mass, 36–50 kDa; reagent grade) was purchased from MP Biomedicals (Solon, OH, USA).

2.2. Animals

Female C57BL/6 mice (5 weeks) were purchased from CLEA Japan (Osaka, Japan). Animals were maintained under standard conditions and used for experimentation after seven days of acclimatization. Use of these mice and the procedures they underwent were approved by the Animal Research Committee of Tottori University (Tottori, Japan).

2.3. Study design

2.3.1. Study of disease severity

Mice ($n = 32$) were randomized into four groups of eight. The NT group was administered tap water and fed a powdered diet (CE-2; CLEA Japan). The DSS group was administered only 3% DSS in tap water and fed a powdered diet. The COS group was administered 3% DSS in tap water and fed a powdered diet containing 2% COS. The GlcN group was administered 3% DSS in tap water and fed a powdered diet containing 2% glucosamine (GlcN). To induce UC, the mice were administered 3% DSS *ad libitum* for 5 days (from day-0 to day-5). The mice in the COS and GlcN groups were also fed a powdered diet with 2% COS or GlcN from day-0 to day-5. Measurements of body weights were undertaken on day-0 and day-5 in all groups. Then, each ratio of the body weights (the percentage of day 5/day 0) was calculated. Colon and blood samplings were done on day-5 in all groups.

2.3.2. Survival study

Mice ($n = 30$) were randomized into three groups of ten. The DSS group was administered only 3% DSS in tap water and fed a powdered diet (CE-2; CLEA Japan). The COS group was administered 3% DSS in tap water and fed a powdered diet containing 2% COS. GlcN group was administered 3% DSS in tap water and fed a powdered diet containing 2% GlcN. To induce UC, the mice were administered 3% DSS *ad libitum* from day 0 to the end of the experiment. Mice in COS and GlcN groups were also fed a powdered diet containing 2% COS or GlcN during the experimental period. The number of days until the mice died was counted. Thereafter, a survival curve was created and median survival time (days) calculated.

2.4. Histopathologic evaluation

The length (cm) and weight (mg) of the colon were measured, and tissue obtained from each colon. Colon tissues were fixed in 10% buffered formalin. Sections (thickness, 3 μm) were made from each sample for histologic observation after staining with hematoxylin and eosin (H&E). Each section was examined microscopically, and histologic scoring was undertaken as described by Ohkawara et al. (2005). In brief, tissue damage was classified using six grades: 0, normal mucosa; 1, infiltration of inflammatory cells; 2, shortening of crypts by less than half of their height; 3, shortening of crypts by more than half of their height; 4, crypt loss; and 5, destruction of epithelial cells. Ten randomly chosen middle-power fields (×100 magnification) for each cross-section were photographed with a digital camera attached to a microscope (Olympus, Tokyo, Japan). Histologic scoring was done in ten fields using three mice in each group. Mean scores for 30 fields were considered as the histologic score for each group.

2.5. Detection of nuclear factor-kappa B (NF-κB) in the colon

Effects of oral administration of COS on NF-κB activation were evaluated in inflamed colons, according to our previous methods (Azuma et al., 2012b). Sections (3 μm) of colon tissue prepared on glass slides were deparaffinized, washed with ethanol and water, and soaked in phosphate-buffered saline (PBS). Then, sections were microwaved with 0.01 M citrate buffer (pH 6.0) for 5 min. Thereafter, they were washed with PBS and incubated with 1% hydrogen peroxide–methanol for 30 min at room temperature. After washing with PBS, sections were incubated with rabbit polyclonal anti-NF-κB p65 antibody (sc-372; 1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 60 min at room temperature, followed by a further wash with PBS. Tissue sections were then visualized by staining with diaminobenzidine tetrahydrochloride (Code No. K3466; Dako, Glostrup, Denmark) and counterstaining with hematoxylin, for 30 min at room temperature.

Then, the extent of NF-κB-positive areas in the colonic epithelium was determined. We used quantitative digital morphometric analyses of NF-κB-positive areas of colonic sections according to a method described previously (Azuma et al., 2012b). In brief, 10 randomly chosen high-power fields (×200 magnification) for each cross-section were photographed with a digital camera attached to a microscope (Olympus). Color wavelengths of the copied image were transformed into digital readings using Lumina Vision software (Mitani, Tokyo, Japan), thereby allowing for quantification of various color wavelengths with pixels as a unit of measure. The original image was used for comparison, and color spectra analyzed; those corresponding to the positive areas of NF-κB were quantified. The percentage of positive areas of NF-κB in the epithelium was calculated by dividing the total pixel area of the positive areas of NF-κB by the total pixel area (which corresponded to the total colonic tissue in the field of view). Colons of three mice were analyzed from each group. The mean score from 30 fields was considered to represent the extent of fibrosis for each group.

2.6. Immunohistochemical (IHC) analyses

Cell staining for myeloperoxidase (MPO; a marker of leukocyte invasion into tissue) (Schindhelm, van der Zwan, Teerlink, & Scheffer, 2009) was undertaken in a routine manner, as described previously (Ohtsuka et al., 2001). Each section was examined microscopically.

For the IHC analyses of cyclooxygenase-2 (COX-2), sections of colon tissue (thickness, 3 μm) prepared on glass slides were deparaffinized, washed with ethanol and water, and soaked in PBS. Then, sections were microwaved with 0.01 M citrate buffer (pH 6.0) for 5 min. Thereafter, they were washed with PBS and incubated with 1% hydrogen peroxide–methanol for 30 min at room temperature. After washing with PBS, blocking was carried out in PBS with 1% bovine serum albumin (BSA) for 30 min at room temperature. Sections were incubated with rabbit monoclonal anti-COX-2 antibody (1:400 dilution; Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C, followed by a further wash with PBS. Subsequently, the envision method was employed for 30 min at room temperature (Dako). Then, tissue sections were visualized by staining with 3.3′-diaminobenzidine (DAB; Dako) and counterstained with hematoxylin. Each section was examined microscopically.

For the IHC analyses of inducible nitric oxide synthase (iNOS), sections of colon tissue (thickness, 3 μm) prepared on glass slides were deparaffinized, washed with ethanol and water, and soaked with PBS. Then, the sections were microwaved with 0.01 M citrate buffer (pH 6.0) for 5 min. Thereafter, they were washed with PBS and incubated with 1% hydrogen peroxide–methanol for 30 min at room temperature. After washing with PBS, blocking was

Table 1
Effects of oral administration of COS on the ratio of body-weight change, colon length, and colon weight/colon length ratio in an experimental model of IBD.

	NT	DSS	COS	GlcN
Ratio of body-weight change (%)	99.8 ± 2.6	89.8 ± 4.6††	95.4 ± 2.8*	95.9 ± 1.4*
Colon length (cm)	7.1 ± 0.3	4.9 ± 0.3††	6.0 ± 0.3**	5.2 ± 0.1
Colon weight/colon length ratio (mg/cm)	26.5 ± 1.5	41.7 ± 2.3††	36.4 ± 2.2*	40.6 ± 1.4

Data are the mean ± S.E. Each ratio of body weight was calculated according to each body weight at day-0 and day-5 (Tukey test).
†† $p < 0.01$ compared with the NT group.
** $p < 0.01$.
* $p < 0.05$ compared with the DSS group.

undertaken in PBS with 1% BSA for 30 min at room temperature. Sections were incubated with rabbit monoclonal anti-iNOS antibody (1:200 dilution; Abcam, Cambridge, UK) overnight at room temperature, followed by another wash with PBS. Subsequently, the envision method was employed for 30 min at room temperature (Dako). Tissue sections were visualized by staining with DAB (Dako) and counterstained with hematoxylin. Each section was examined microscopically.

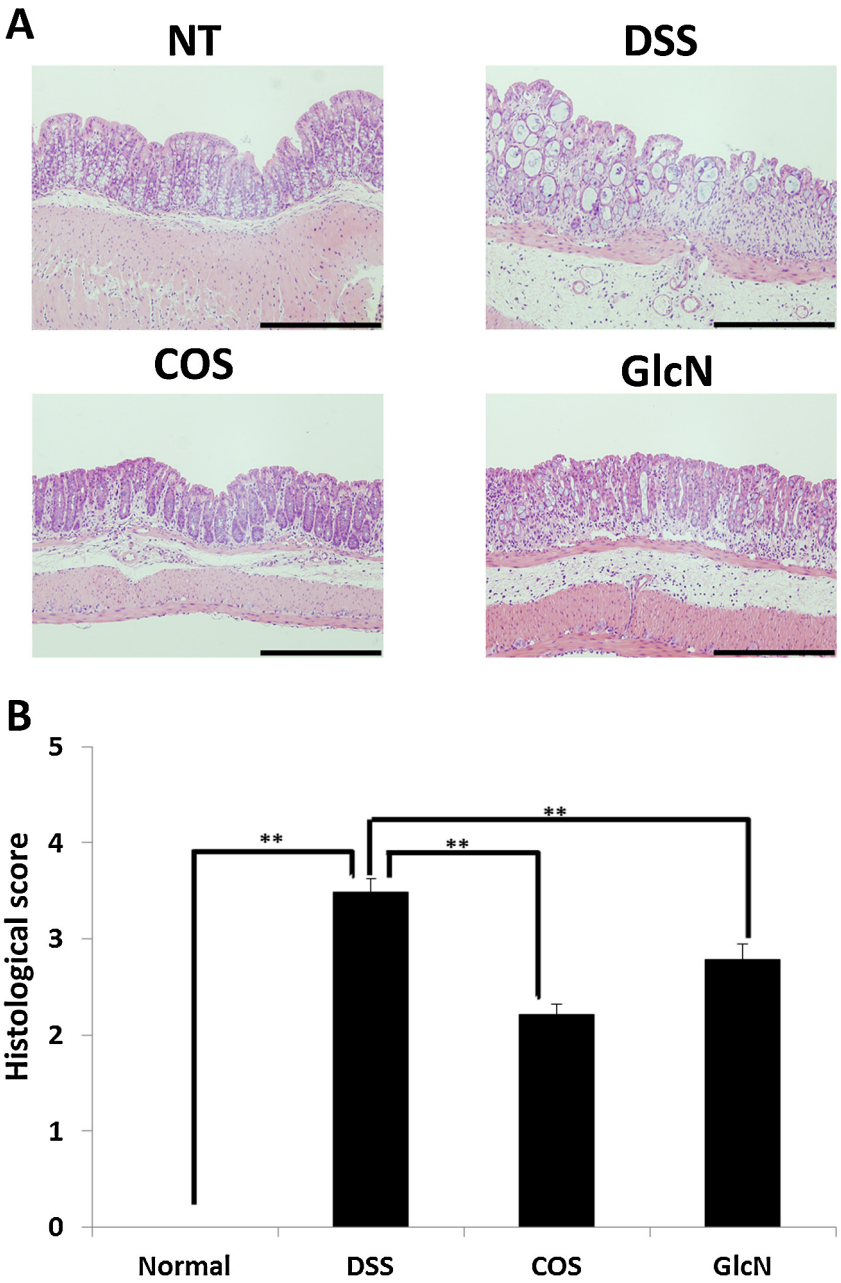

Fig. 1. Effects of oral administration of COS on histopathologic change in an experimental model of IBD. (A) Sections of colon tissue were stained with hematoxylin and eosin. Data are for one-mouse each from the NT, DSS, COS and GlcN groups. Bar = 200 μm. (B) Data are the mean ± S.E. of 30 fields/ × 100 magnification field in each group (Steel-Dwass test). ** $p < 0.01$.

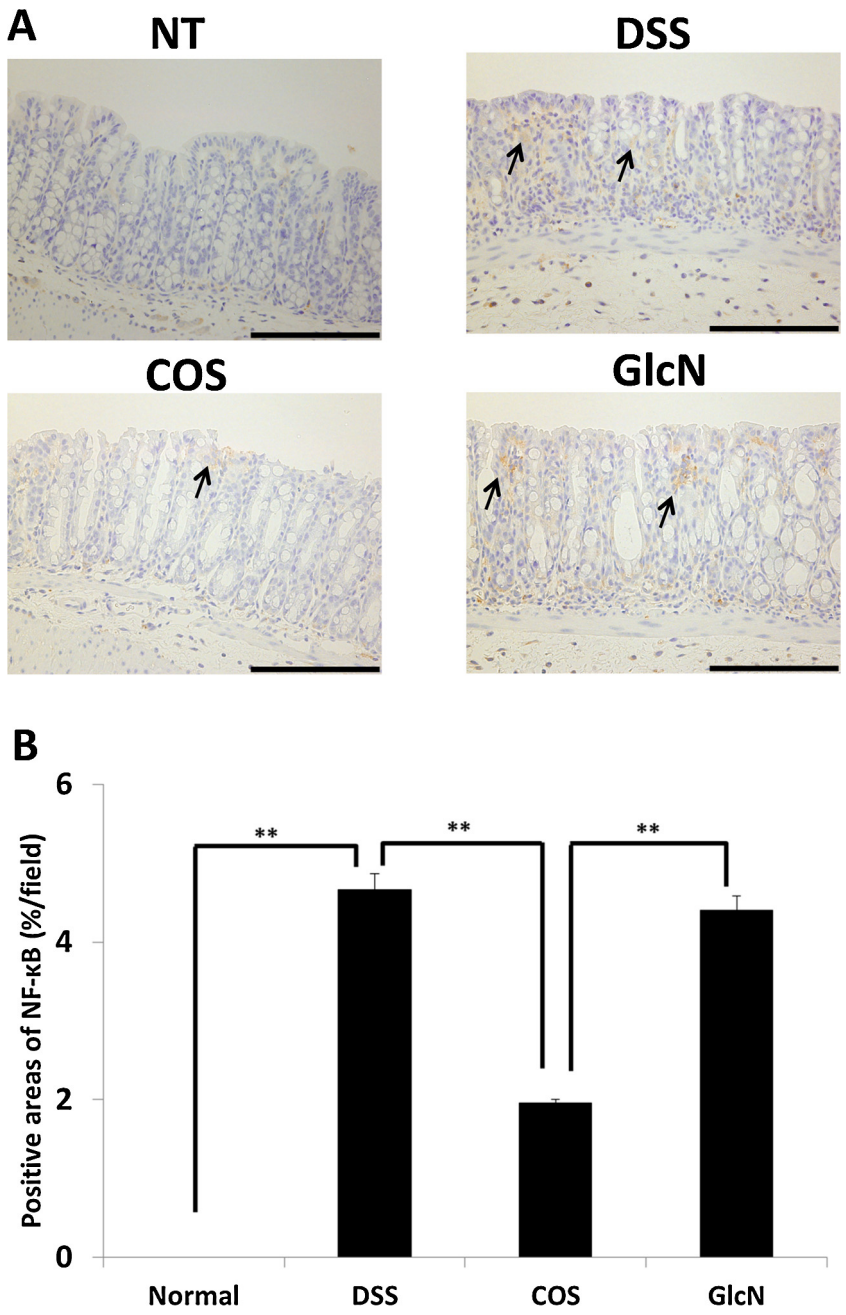

Fig. 2. Effects of oral administration of COS on NF-κB activation in the colon in an experimental model of IBD. (A) Positive areas of NF-κB were shown by arrows. Data are for one-mouse each from NT, DSS, COS and GlcN groups. Bar = 100 μm. (B) Data are the mean ± S.E. of 30 fields/×100 magnification field in each group (Steel-Dwass test). ** $p < 0.01$.

2.7. Measurement of serum levels of cytokines

Serum levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and monocyte chemotactic protein 1 (MCP-1/CCL2) were quantified by a sandwich enzyme-linked immunosorbent assay (ELISA) using a mouse (TNF)-α, IL-6 and MCP-1 ELISA kit (Quantikine®, R&D Systems, Minneapolis, MN, USA) according to manufacturer instructions.

2.8. Statistical analyses

Data are the mean ± standard error (SE). Statistical analyses were carried out using one-way ANOVA followed by the Tukey–Kramer test or Steel-Dwass test. The log-rank test was done to evaluate the survival of mice. $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Effects of oral administration of COS on change in body weight, colon length, and colon weight/colon length ratio in an experimental model of IBD

In the DSS group, the ratio of body-weight change was decreased significantly compared with that of the NT group ($p < 0.01$) (Table 1). In COS and GlcN groups, the decrease in body-weight change was suppressed significantly compared with that of the DSS group ($p < 0.05$).

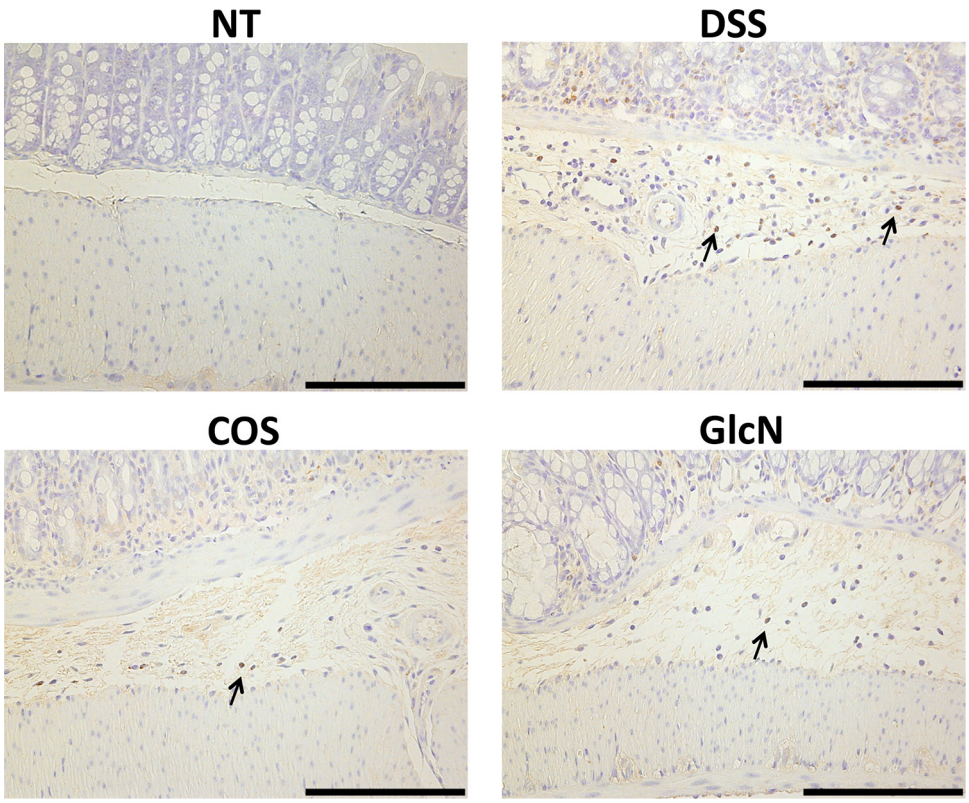

Fig. 3. Effects of oral administration of COS on MPO activation in the colon in an experimental model of IBD. The immunohistochemistry of MPO in the colon is shown. Positive areas of MPO are shown by arrows. Data are for one-mouse each from NT, DSS, COS and GlcN groups. Bar = 100 μm.

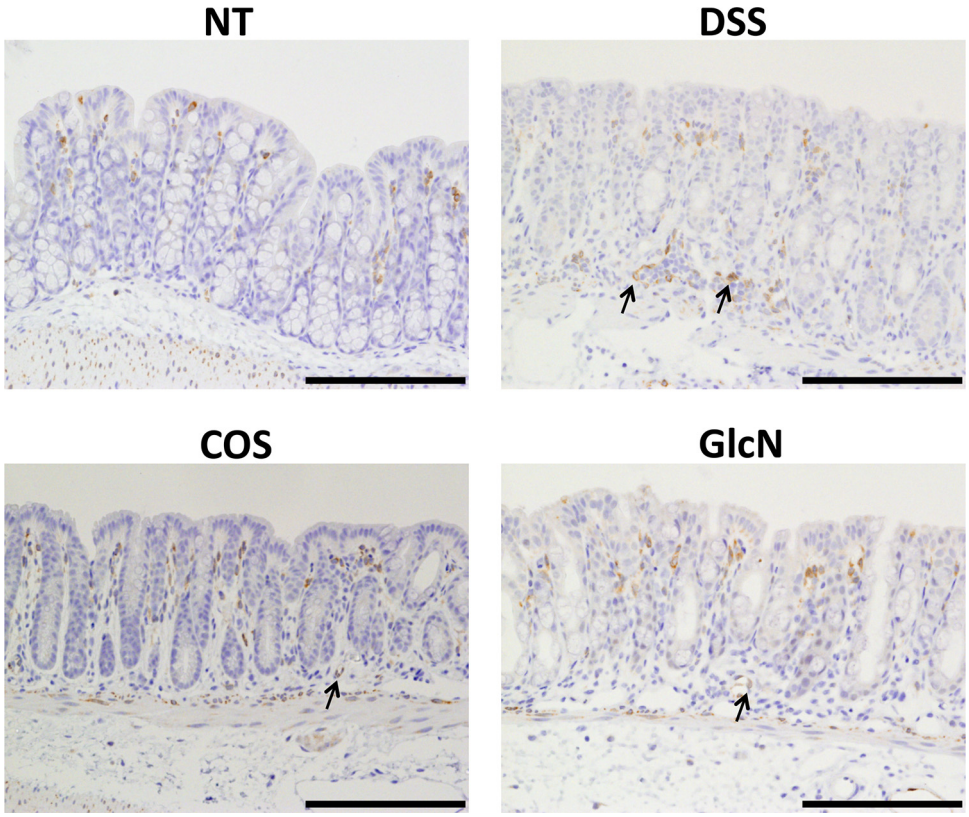

Fig. 4. Effects of oral administration of COS on COX-2 activation in the colon in an experimental model of IBD. The immunohistochemistry of COX-2 in the colon is shown. Positive areas of COX-2 are shown by arrows. Data are for one-mouse each from NT, DSS, COS and GlcN groups. Bar = 100 μm.

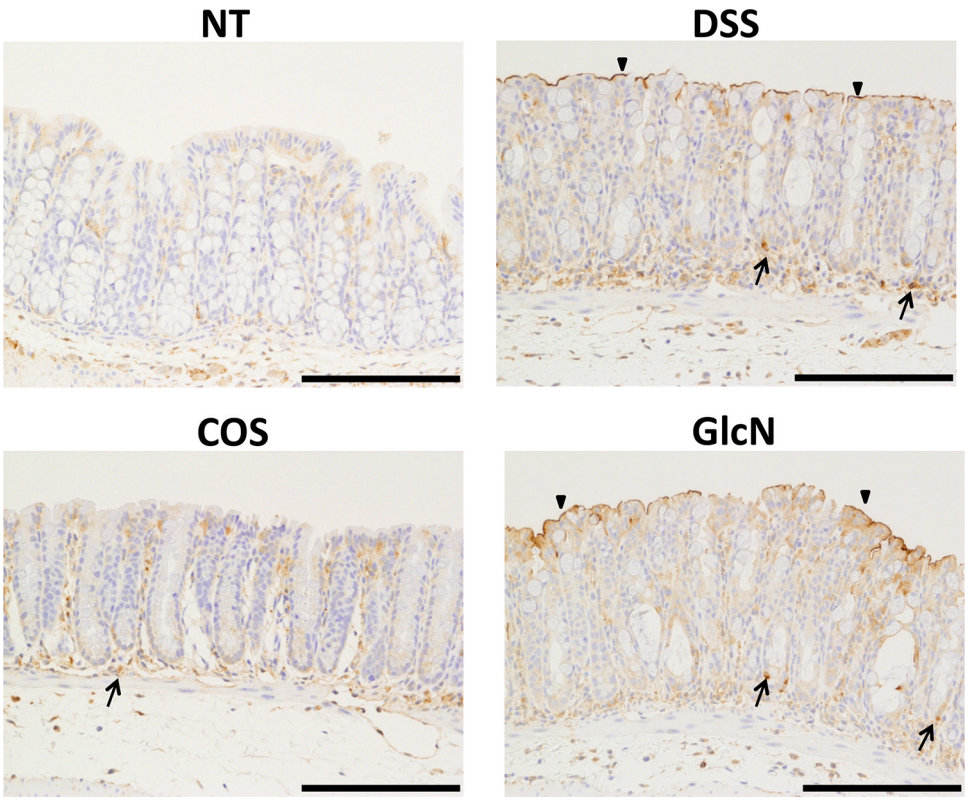

Fig. 5. Effects of oral administration of COS on iNOS activation in the colon in an experimental model of IBD. The immunohistochemistry of iNOS in the colon is shown. Positive areas of iNOS are shown by arrows and arrowheads. Data are for one-mouse each from NT, DSS, COS and GlcN groups. Bar = 100 μm.

In the DSS group, colon length was decreased significantly compared with that of the NT group ($p < 0.01$). In the COS group, the decrease in colon length was suppressed significantly compared with that of the DSS group ($p < 0.01$). There was no change in the colon lengths between DSS and GlcN groups.

In the DSS group, the colon weight/colon length ratio was increased significantly compared with that of the NT group ($p < 0.01$). In the COS group, the increase in the colon weight/colon length ratio was suppressed significantly compared with that of the NT group ($p < 0.05$). There was no change in the colon weight/colon length ratio between DSS and GlcN groups.

Administration of 3% DSS induces shortening of colon length and increases the colon weight/colon length ratio in C57BL/6 mice (Melgar, Karlsson, & Michaëlsson, 2005). The present study suggested that oral administration of COS suppressed loss in body weight, shortened colon length, and increased the colon weight/colon length ratio. Moreover, oral administration of COS prolonged the survival time in this experimental model of IBD.

3.2. Effects of oral administration of COS on histopathologic changes

In the DSS group, erosions, shortening or destruction of crypts, and severe edema were observed (Fig. 1A). In the GlcN group, some erosion was observed, and shortening/destruction of crypts was suppressed; moreover, edema was suppressed slightly (Fig. 1A). In the COS group, shortening/destruction of crypts and edema were suppressed markedly (Fig. 1A).

The results of histopathologic scoring are shown in Fig. 1B. In the DSS group (3.5 ± 0.1), the histologic scores were significantly higher than those of the NT group (0.0 ± 0.0; $p < 0.01$). Scores in the GlcN group (2.8 ± 0.2) were significantly lower than those of the DSS group ($p < 0.01$). In the COS group, the score (2.8 ± 0.2) were significantly decreased compared to that of the DSS group ($p < 0.01$). Moreover, the score of the COS group were lower than that of the GlcN group, however, there was no significant difference.

Microscopic observation of DSS-induced colitis in C57BL/6 mice has shown infiltration of macrophages, lymphocytes, and plasma cells into the mucosa and submucosa as inflammation progresses (Melgar et al., 2005). In the COS group, colon damage was suppressed significantly compared with that in the DSS group. These results suggest that oral administration of COS suppresses colon damage in our experimental model of IBD. Reports have suggested that GlcN has anti-inflammatory effects in experimental models of IBD (Yomogida et al., 2008). GlcN also suppressed colon damage in the present study, but its suppressive effect was not as great as that of COS.

3.3. Effects of oral administration of COS on NF-κB levels in the colonic epithelium

The results of IHC detection of NF-κB are shown in Fig. 2A. In DSS and GlcN groups, many positive areas of NF-κB in epithelial cells were observed. In the COS group, the number of positive areas of NF-κB in the epithelial cells was markedly less than in DSS and GlcN groups.

To evaluate the effect of COS on NF-κB activation in epithelial cells, we analyzed digital images. In the DSS group ($4.7 \pm 0.2\%$ per field), there were significantly more positive areas of NF-κB than in the NT group ($0.0 \pm 0.0\%$ per field; $p < 0.01$). In the COS group ($2.0 \pm 0.1\%$ per field), there were significantly fewer positive areas of NF-κB than in DSS and GlcN groups ($4.4 \pm 0.2\%$ per field) ($p < 0.01$; Fig. 2B).

In the COS group, the number of positive areas of NF-κB staining in the colon was decreased significantly compared with that of the DSS group. NF-κB occupies a pivotal position in several signaling

pathways involved in innate immunity. NF-κB stimulates expression of COX-2, prostaglandin E2, and pro-inflammatory cytokines (IL-6, TNF-α, and MCP-1) (Karrasch & Jobin, 2008). NF-κB is the critical transcription factor needed to express genes associated with pro-inflammatory responses (Elson et al., 2005).

3.4. Effects of oral administration of COS on immunohistochemistry in the colonic epithelium

Results of IHC detection of MPO are shown in Fig. 3. In the NT group, no MPO-positive cells were observed. In the DSS group, many MPO-positive cells were observed. In COS and GlcN groups, there were fewer MPO-positive cells than in the DSS group.

MPO is a marker of oxidative stress. High MPO activities were observed in a DSS-induced model of UC (Naito, Takagi, & Yoshikawa, 2007; Schindhelm et al., 2009). Oxidative stress has important roles in the pathogenesis of IBD (Kruidenier, Kuiper, Lamers, & Verspaget, 2003). In the present study, oral administration of COS suppressed MPO activations in the colon. A possible anti-inflammatory mechanism of COS in UC might come from the strong anti-oxidative effects. However, information regarding the relationship between COS and anti-oxidative stress is limited. To understand the anti-inflammatory mechanism of COS, studies focusing on the anti-oxidative effects of COS must be carried out *in vitro* and *in vivo*.

Results of IHC detection of COX-2 are shown in Fig. 4. In the DSS group, some positive areas of COX-2 were observed. In COS and GlcN groups, the number of positive areas of COX-2 was decreased slightly compared with those in the DSS group.

Cyclooxygenases are the enzymes responsible for the metabolism of arachidonic acid, converting it into prostaglandins. These products influence many biological processes, including homeostasis and inflammation (Grisham et al., 2002). In fact, COX-2 expression is increased mainly during inflammatory processes and cell transformation (Araujo, Soeiro, Fernandes, & Serrano, 2005).

Results of IHC detection of iNOS are shown in Fig. 5. In DSS and GlcN groups, many positive areas of iNOS were observed in the colonic epithelium. In the COS group, the number of positive areas of iNOS in the colonic epithelium was markedly less than those of DSS and GlcN groups.

It has become increasingly clear that nitric oxide (NO) overproduction by iNOS is deleterious to intestinal function (Cross & Wilson, 2003; Hogaboam, Jacobson, Collins, & Blennerhassett, 1995). iNOS is considered to be an important determinant of colonic damage (Rosillo, Sanchez-Hidalgo, Cárdeno, & de la Lastra, 2011). Hence, sustained high production of NO mediated by iNOS may have a role in the pathogenesis of IBD and induction of experimental colitis in the colon (Hogaboam et al., 1995).

3.5. Effects of oral administration of COS on serum levels of cytokines

Results of measurement of serum levels of cytokines are shown in Fig. 6. In the DSS group (221.4 ± 15.3 pg/mL), the serum level of TNF-α was significantly higher than that of the NT group (37.3 ± 6.1ϒpg/mL; $p < 0.01$). In COS (63.5 ± 10.7 pg/mL) and GlcN (100.7 ± 16.6 pg/mL) groups, serum levels of TNF-α were significantly lower than that of the DSS group ($p < 0.01$). However, there was no significant difference between COS and GlcN groups.

In the DSS group (43.9 ± 12.9 pg/mL), the serum level of Il-6 was significantly higher than that of the NT group (3.9 ± 1.0 pg/mL; $p < 0.01$). In COS (17.0 ± 2.2 pg/mL) and GlcN (16.8 ± 3.5 pg/mL) groups, serum levels of IL-6 were significantly lower than that of the DSS group ($p < 0.01$). However, there was no significant difference between COS and GlcN groups.

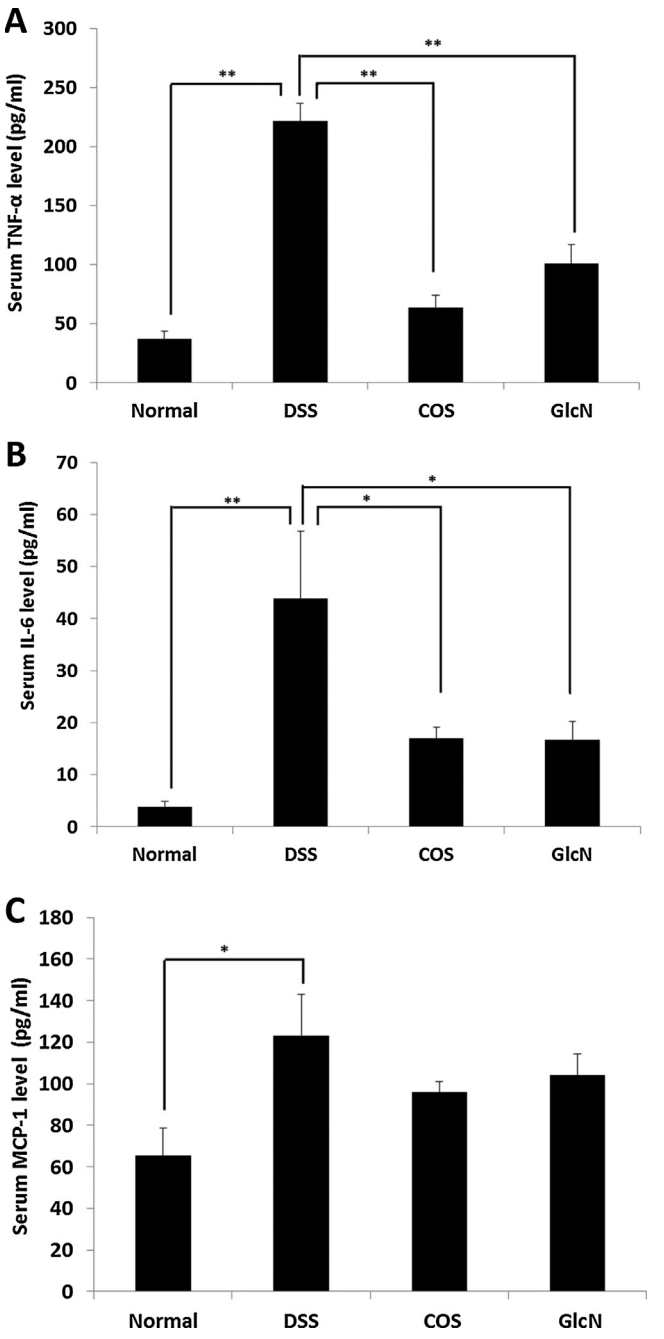

Fig. 6. Effects of oral administration of COS on serum levels of cytokines in an experimental model of IBD. Data are the mean ± S.E. in each group (Tukey–Kramer test). ** $p < 0.01$, * $p < 0.05$.

In the DSS group (123.2 ± 19.9 pg/mL), serum levels of MCP-1 were significantly higher than that of the NT group (65.4 ± 13.3 pg/mL; $p < 0.05$). In COS (95.9 ± 5.0 pg/mL) and GlcN (104.1 ± 10.4 pg/mL) groups, serum levels of MCP-1 were slightly lower than that of the DSS group. However, there was no significant difference among DSS, COS and GlcN groups.

Moreover, pro-inflammatory cytokines (IL-6, TNF-α, MCP-1) play a part in inflammatory conditions such as colitis by triggering leukocyte activation and accumulating in tissues (Martín, Villegas, Sánchez-Hidalgo, & de la Lastra, 2006; Sasaki et al., 2004). Our results suggest that one possible mechanism underlying the anti-inflammatory effects of oral administration of COS is suppression of inflammatory processes, including expression of NF-κB, COX-2, iNOS, and pro-inflammatory cytokines.

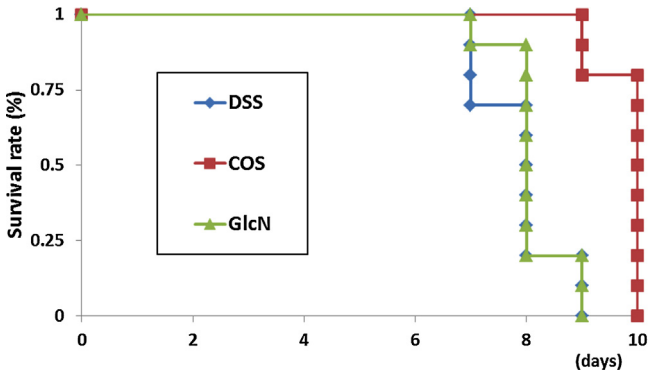

Fig. 7. Effects of oral administration of COS on survival time in an experimental model of IBD. $n = 10$ in each group. The survival time in the COS group was significantly longer compared to the control and GlcN groups ($p < 0.05$, Long-Rank test).

Oral administration of GlcN also showed suppression of COX-2 expression and serum levels of pro-inflammatory cytokines, but there was no effect on the expression of NF-κB in the colon. Some reports have suggested that GlcN suppresses expression of inflammatory cytokines (at least in part) *via* attenuation of mitogen-activated protein kinase (MAPK) activation *in vitro* (Tsai, Lee, Kou, & Wu, 2009; Wu et al., 2010). Those results might suggest that the anti-inflammatory effects of GlcN arise from suppression of MAPK activation.

3.6. Effects of oral administration of COS on survival time in an experimental model of IBD

Effects of oral administration of COS on survival time in an experimental model of IBD are shown in Fig. 7. Survival time of mice in the COS group was increased significantly compared with that of mice in NT and GlcN groups (log-rank test, $p < 0.05$). Median survival time was 8 days in NT and GlcN groups and 10 days in the COS group.

Yousef et al. (2012) reported that intraperitoneal injection of COS suppressed colonic inflammation and prolonged survival time in an experimental model of IBD. Studies describing the anti-inflammatory effects of COS by oral administration have not been published. This is the first study to evaluate the anti-inflammatory effects of COS by oral administration.

Several treatments are used for IBD, such as 5-aminosalicylic acid (5-ASA) drugs (*e.g.*, sulfasalazine or balsalazide), immunomodulators (*e.g.*, azathioprine, 6-mercaptopurine), methotrexate, and biologic therapies that target TNF-α or IL-6 (Morrison et al., 2009; Nakamura, Honda, Mizutani, Akiho, & Harada, 2006). However, 5-ASA drugs are expensive and immunomodulators and biologic therapies increase the risk of serious infection (Morrison et al., 2009). It has been reported that some nutritional supplements are beneficial for IBD, including amino acids (Coëffier, Marion-Letellier, & Déchelotte, 2010), omega-3 fatty acids (Rajendran & Kumar, 2010), and probiotics (Vanderpool, Yan, & Polk, 2008). Our results suggest that COS have potential as new functional foods for IBD patients.

4. Conclusion

The present study revealed that oral administration of COS can inhibit colonic inflammation and reduced tissue injury in an experimental model of IBD. Oral administration of COS inhibited inflammation in the colonic mucosa by suppression of MPO activation in inflammatory cells and activation of NF-κB, COX-2 and iNOS. Oral administration of COS also suppressed serum levels of pro-inflammatory cytokines in this model. Moreover, oral administration of COS prolonged the survival of mice in this model. These results suggest that COS could be new functional foods for IBD patients.

Acknowledgments

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