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Research Article
Chitooligosaccharides Downregulate TLR4/NF-κB/COX-2 Signaling Cascade in Dextran Sulfate Sodium-treated Mice: A Potential Mechanism for the Anti-colitis Effect

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Abstract

Background and Objective: The anti-inflammatory properties of chitooligosaccharides (COS), a new class of marine-derived natural products and functional foods have been well established, but the underlying mechanisms remain unclear. The aim of the present study is to investigate the effect and potential mechanisms of COS on colonic inflammation in a mouse model of experimental colitis induced by 3.5% Dextran Sulfate Sodium (DSS). **Materials and Methods:** Animals were randomly divided into 6 groups of 10 mice each: Normal group, model control COS treatment groups: Administered with COS at low (125 mg kg^{-1}), middle (250 mg kg^{-1}) and high (500 mg kg^{-1}) oral administration dose once per day, respectively and positive control group: Salazosulfapyridine 50 mg kg^{-1}. The mRNA and protein expression of toll-like receptor 4 (TLR4), nuclear factor kappa B (NF-κB) and cyclooxygenase (COX)-2 was measured in colon tissues using real-time PCR and immunofluorescence, respectively. **Results:** Oral administration of COS dose-dependently decreased bloody diarrhea event rate and the Disease Activity Index (DAI), attenuated the loss of body weight, shortening of colon length induced by DSS. Histological damage scores of the colon and colonic prostaglandin (PG)E_2 content in DSS-treated mice were reduced by COS, suggesting the preventive effect of COS on the colonic inflammation. Importantly, it was found that the anti-colitis effect shown by COS appear to be related to their capacity to downregulate the expression of TLR4/NF-κB pathway, a canonical proinflamatory signalling, together with its downstream COX-2. **Conclusion:** The COS administration prevented the DSS-induced activation of TLR4/NF-κB/COX-2 signaling cascade in mouse colon. These finding could give insight into the further evaluation of COS as a food supplement or an alternative agent in the control of ulcerative colitis.

Key words: Chitooligosaccharides, ulcerative colitis, colonic inflammation, TLR4, NF-κB, COX-2

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ulcerative Colitis (UC) is one of the two primary types of inflammatory bowel disease and has a high incidence and a prevalence rate around the world. The main characteristic of UC is recurrent uncontrolled inflammation of the colon, which causes acute abdominal pain, severe diarrhea, bloody stool symptoms, weight loss thus impairs patient's quality of life. And the risk of colorectal cancer development has been found be much higher in patients with UC than that in the general population[1,2]. The initiation and maintenance of colonic inflammation, which is characterized by transmural infiltration of leukocytes in the mucosa, overproduction of inflammatory cytokines etc. are essential for subsequent mucosal disruption and ulceration, thus play a key role in the pathogenesis of UC, especially in the early stage of the disease[3,4]. Accordingly, treating UC mainly depends on the use of medications that can inhibit the inflammation in the colon and control the symptoms. Some anti-inflammatory drugs such as 5-aminosalicylic acid and steroid hormone have been commonly used to treat UC. However, traditional anti-inflammatory pharmaceutical compounds often have undesirable side effects, which would decrease patient compliance and make the condition worse[5,6]. Therefore, it is urgent to explore new anti-inflammatory drugs with greater safety for reducing UC.

Chitooligosaccharides (COS) produced from chitosan in the shell of crustaceans and marine zoo-plankton are a new class of marine-derived natural products possessing potent anti-inflammatory properties and have exhibited protective effects in various inflammatory conditions[7,8]. The COS at a single dose of 500 mg kg^{-1} were believed suitable to treat acute inflammation cases following the carrageenan-induced paw edema method[9]. And the anti-inflammatory activity of COS has been confirmed by a series of studies *in vivo* and *in vitro*, which reported that COS could attenuate the inflammation in animals with inflammation-related osteoporosis[10], experimental autoimmune anterior uveitis[11], retinal ischemia and reperfusion injury[12] and in lipopolysaccharide -induced human umbilical vein endothelial cells[13], RAW264.7 macrophage cells[14,15], porcine iliac artery endothelial cells[16], microglia[17] and so on. The oral intake of COS in elderly adults decreased the serum levels of inflammatory cytokines[8]. Especially, nontoxity, easy solubility, high absorptivity and low cost of COS have attracted the interest of researchers to utilize COS for various clinical applications[18]. However, the complete mechanism of anti-inflammatory action of COS remains unknown. In the present study, using Dextran Sulfate Sodium (DSS)-induced colitis in mice, a well-established experimental model that resembles many of the signs and symptoms of human UC[19], it was evaluated the effect of COS on the colonic inflammation. Most importantly, the primary focus is on the possible molecular mechanisms for COS' anti-inflammatory effect.

MATERIALS AND METHODS

Animal: Eight week-old female C57BL/6J mice weighting 20±2 g were obtained from the Laboratory Animal Center of Hubei Province (Wuhan, China) and maintained at a room temperature of 25°C and a relative humidity between 50 and 60%, under a standard 12 h light/12 h dark cycle. All animal protocols were approved by the Laboratory Animal Ethical Committee of Three Gorges University.

Chemical: The COS (Qingdao BZ Oligo Biotech Co., Ltd., China) with the degree of deacetylation of 90% and average molecular weight of 1500 Da were prepared from chitosan in crustaceans shell by enzymatic hydrolysis. The purity has been determined by HPLC and achieved 90% at least.

Induction of colitis and treatment with COS: After fed in the facility for 1 week, all animals were randomly divided into 6 groups of 10 mice each; (1) Normal group, (2) Model control: Orally administered with the same volume of vehicle control (PBS), (3-5) COS treatment groups: Administered with COS at low (125 mg kg^{-1}), middle (250 mg kg^{-1}) and high (500 mg kg^{-1}) oral administration dose once per day, respectively and (6) Positive control group: Salazosulfapyridine (SASP) was administrated orally at a dosage of 50 mg kg^{-1} b.wt., once per day. After COS pretreatment for 1 week, mice in 2-6 groups were given 3.5% (w/v) DSS (molecule weight 36,000-50,000, MP Biomedicals, Aurora, USA) in drinking water for 7 consecutive days to induce experimental colitis. Normal (untreated) mice were given normal drinking water.

Assessment of the severity of the induced colitis: Body weight and bloody diarrhea were measured daily. The daily weight changes were calculated as the percent of the initial weight. The data on bloody diarrhea is presented as the percentage of mice in each group showing gross blood in feces and at the anus. Disease severity was evaluated using the Disease Activity Index (DAI), a combined score of stool consistency, fecal occult blood and loss of body weight, as described previously[20]. Mice were sacrificed by cervical dislocation 1 h after the last administration. Colonic damage was evaluated from the colon length. The entire colon from the ileocecal junction to the anal verge was resected and the length was measured with calipers.

Histological analysis: Tissue samples of the distal colon were stained routinely with hematoxylin and eosin (H and E) for histological examination. Two independent pathologists evaluated the colonic damage using a histological scoring system for assessing epithelial structure and inflammatory cell infiltration as previously described[21].

Measurement of colonic PGE_2 levels: As described previously[22], the tissues of the distal colon were homogenized in PBS (pH 7.4) containing 1 mM EDTA and 10 μM indomethacin for 10 min on ice, then centrifuged at 8000 g for 10 min at 4°C. The PGE_2 levels in the supernatant were determined according to the ELISA kit protocol (R and D Systems, MN, USA).

Real-time PCR: Total RNA from colon tissue was extracted using a TRIzol reagent according to the manufacturer's instructions. After the photometrical determination of RNA concentration and purity, cDNA was generated from total RNA using the all-in-one™ first-strand cDNA synthesis kit (GeneCopoeia, USA). The TLR4 primer pair: 5-CGC TTT CAC CTC TGC CTT CAC TAC AG-3 and 5-ACA CTA CCA CAA TAA CCT TCC GGC TC-3, NF-κB p65 primer pair: 5-GCT TTG CAA ACC TGG GAA TA-3 and 5-TCC GCC TTC TGC TTG TAG AT-3; COX-2 primer pair: 5-ACG CTT CTC CCT GAA GCC GTA C-3 and 5-GTA GAG GGC TTT CAA TTC TGC AGC C-3; β-actin primer pair: 5-GAT TAC TGC TCT GGC TCC TAG C-3 and 5-GAC TCA TCG TAC TCC TGC TTG C-3. Real-time PCR was carried out in an applied biosystems StepOnePlus™ real-time PCR system. Levels of TLR4, NF-κBp65 and COX-2 mRNA were normalized against β-actin mRNA. All experiments were repeated 3 times.

Immunofluorescence: Colon tissues for immunofluorescence were embedded in paraffin. Then, 5 μm-thick sections were deparaffinized, rehydrated, blocked in 10% donkey serum for 30 min and incubated with anti-TLR4, anti-NF-κBp65 or anti-COX-2 (Cell signaling, USA) primary antibodies overnight at 4°C. After washing 3 times with PBS, sections were incubated with the corresponding secondary antibodies (Cell signaling, USA) in the dark. After washes sections were stained with 4, 6-diamino-2-phenyl indole (DAPI) (0.5 mg mL^{-1}, Sigma, St., Louis, MO) to visualize nuclei. Images were captured using an Olympus fluorescent microscope (Olympus, Center Valley, PA).

Statistical analysis: Data were expressed as Mean±Standard Deviation (SD) and analyzed with statistical software package SPSS 11.5. Differences between groups were analyzed by Student's t-test analysis. Results of body weight, colon length, DAI scores and histologic scores were analyzed using an ANOVA test. Chi-square test was used to analyze differences of the occurrence of bloody diarrhea. A $p<0.05$ was considered significant.

RESULTS

COS prevented DSS-induced experimental colitis in mice: The protective effect of oral administration of COS on experimental colitis was assessed using body weight loss, bloody diarrhea event rate DAI and colon lengths. The reduction of body weight induced by DSS administration started on day 1 compared with the normal mice ($p<0.01$, Fig. 1a). The COS at the doses of 125, 250 and 500 mg kg^{-1} suppressed DSS-induced body weight loss were found starting on day 5, 4 and 4, respectively, lasted up to day 7. After 7 days of treatment, COS (125, 250 and 500 mg kg^{-1}) significantly increased the body weight of mice by 12.3, 20.5 and 23.3% of DSS group, respectively ($p<0.01$). Figure 1b shows gross bleeding in stool occurred around day 4-6 after starting DSS administration. But bloody diarrhea event rate was significantly decreased in SASP or 50 mg kg^{-1} COS treated group ($p<0.05$, $p<0.01$). The DAI, a combined index integrated with stool consistency, fecal occult blood and loss of body weight was measured on day 7 of treatment. It was found that treatment with COS significantly suppressed DSS-induced DAI increase in a dose-dependent manner with statistically significant difference at the doses of 250 and 500 mg kg^{-1} ($p<0.01$, Fig. 1c). In addition, colon length is inversely related to the severity of DSS-induced colitis. In the presence of COS at the dose of 125 or 250 mg kg^{-1}, significant colon shortening induced by DSS was improved ($p<0.01$, Fig. 1d). These data suggested COS could reduce the disease severity of colitis in mice and the effects of COS were similar to those of the reference drug SASP (50 mg kg^{-1} day).

COS attenuated DSS-induced colonic inflammation: Colonic inflammation is an important feature during the course of UC[2]. To consolidate the effect of COS on UC, the severity of colonic inflammation was evaluated based on histological examination and the measurement of PGE_2 levels in colon.

Histological analysis of the distal colon specimens showed that DSS treatment resulted in typical inflamed signs, including extensive epithelial disintegration, mucosal ulcerations, loss of goblet cell and infiltration of massive inflammatory cells in mucosa and sub-mucosa, as compared

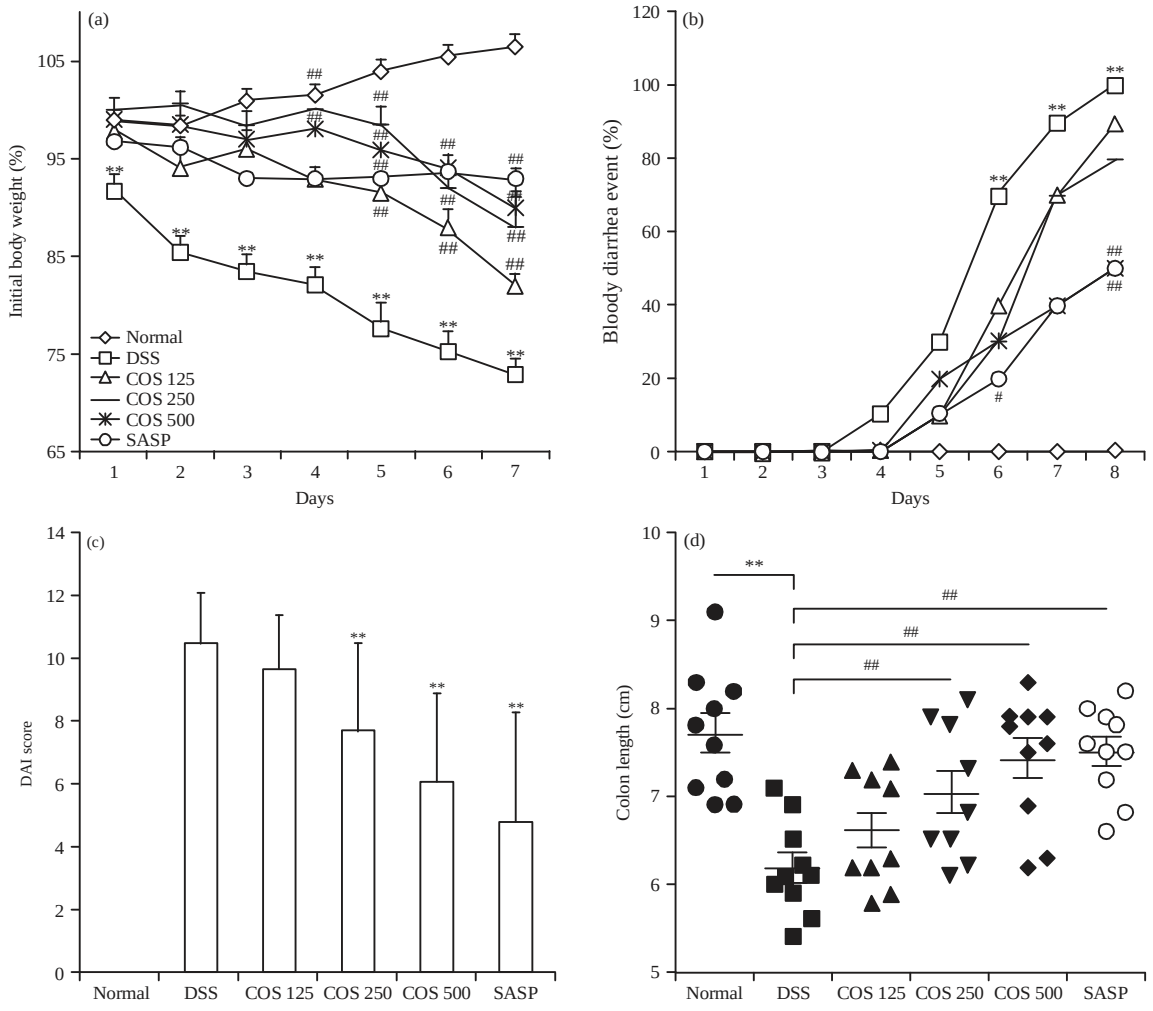

Fig. 1(a-d): Effect of COS on body weight changes, bloody diarrhea, DAI scores and colon length in mice following DSS induction of colitis. (a) The daily weight changes were calculated as the percent of the initial weight, (b) The data on bloody diarrhea is presented as the percentage of mice in each group showing gross blood in feces and at the anus, (c) DAI scores at day 7 of treatment with DSS and (d) Assessment of colon shortening. Values were expressed as the Mean±SD, n = 10, **p<0.01 vs the normal group and $^{\#}$p<0.05, $^{\#\#}$p<0.01 vs DSS model group

to normal mice. Interestingly, COS or SASP administration to DSS-treated mice significantly protected epithelial structure, reduced inflammatory cell infiltration, improved crypt distortion in colon tissues (Fig. 2a). And COS treatment at middle or high dose (125 or 250 mg kg^{-1}) resulted in a significant reduction of the histological damage scores induced by DSS (p<0.01, Fig. 2b).

Intramucosal levels of PGE_2 are substantially increased in UC[23] and can be considered as a marker of colonic inflammation activity in patients with UC[24]. Figure 2c shows DSS induced a significant increase in the colonic PGE_2 content reaching 3.7 folds as compared to the normal group (p<0.01). Administration of SASP resulted in a decrease of PGE_2 concentration by about 52% compared to DSS group (p<0.01). Likewise, colonic PGE_2 content was significantly reduced in the mice treated with COS (250, 500 mg kg^{-1}) by 20 and 44%, respectively as compared to animals only received DSS (p<0.05, p<0.01).

Taken together, the data presented here supported that COS could attenuate DSS-induced colonic inflammation.

COS downregulated the expression of TLR4, NF-κB and COX-2 in the colons of DSS-treated mice: Based on the above finding about the preventive effect of COS on DSS-induced colonic inflammation, it was further explored the potential molecular mechanism. The mRNA and protein expression of

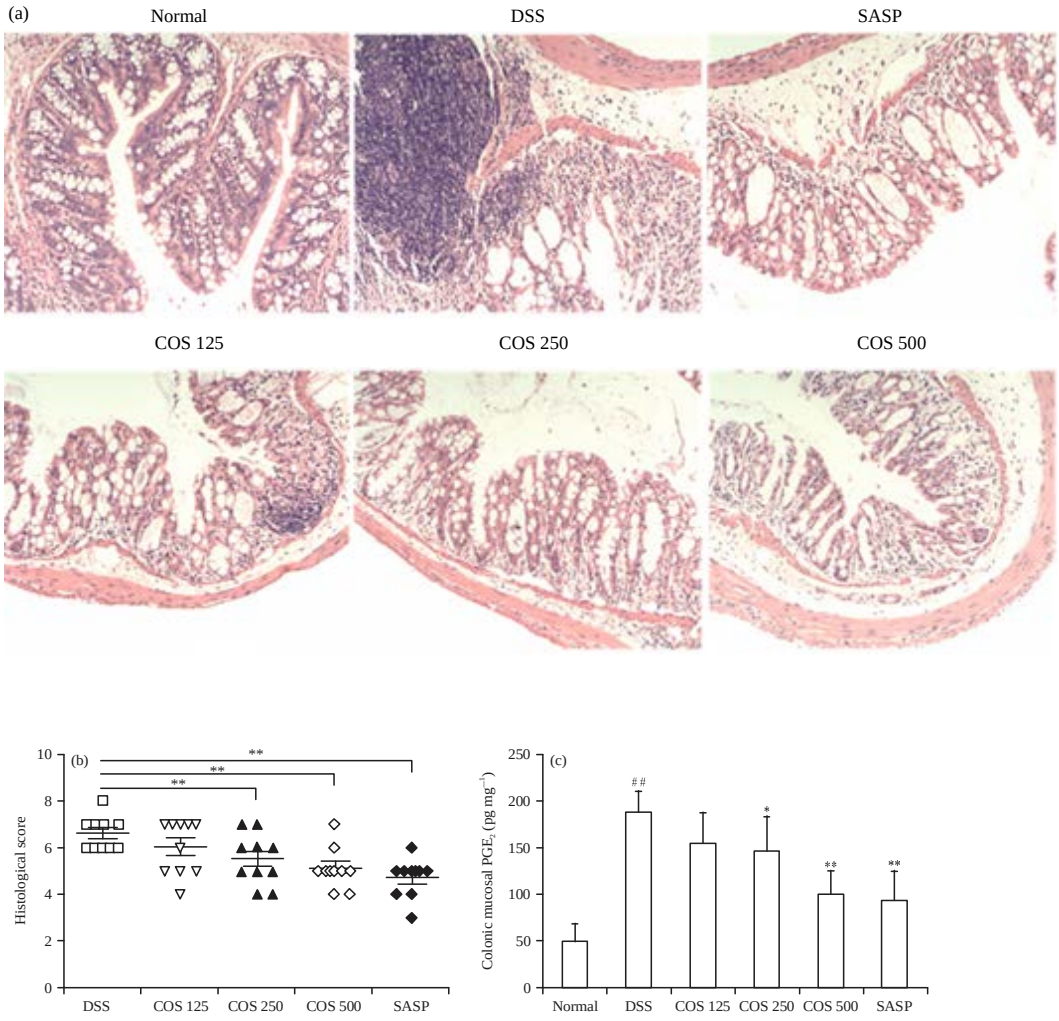

Fig. 2(a-c): Effect of COS on colonic inflammation in mice following DSS induction of colitis. (a) Representative H and E-stained colon sections, (b) Histology scores and (c) Colonic PGE_2 levels. Values were expressed as the Mean±SD of n = 10 mice in each group, ##p<0.01 vs the normal group and *p<0.05,**p<0.01 vs DSS model group

COX-2, a key enzyme mediating PGE_2 production was measured in colon tissues using real-time PCR and immunofluorescence, respectively. And it way found the upregulated expression of COX-2 in the colons of DSS-treated mice were significantly reduced in middle and high dose COS groups (p<0.01, Fig. 3a, 4).

Remarkably, COX-2 expression is closely related to toll-like receptor 4 (TLR4)/NF-κB signaling in the gut, especially in the setting of DSS colitis[25,26]. As a key receptor in gut innate immunity, TLR4 was found over-expressed in inflamed colon of UC patients[27]. And TLR4-induced signalling further leads to the activation of NF-κB, followed by the expression of an array of subsequent genes (e.g., COX-2) involved in the inflammatory signalling cascade, thus mediates the pathogenesis of colitis[26]. The suppression of TLR4/NF-κB signaling pathway has been believed as one of the important mechanisms involved in the therapeutic effects of many anti-inflammatory agents against UC[26,28]. Based on the key role of TLR4 and its downstream NF-κB in colonic inflammation, it was focused the effect of COS on TLR4/NF-κB pathway in DSS-induced murine colitis. Figure 3b, c and 4 showed that DSS-treated mice exhibited elevated mRNA and protein expression of TLR4 and NF-κB p65. However, administration of COS reduced TLR4 and NF-κB p65 expression in DSS-induced colitis. The data indicated that COS might provide the protection from DSS-induced colitis through the suppression of TLR4/NF-κB/COX-2 signaling cascade.

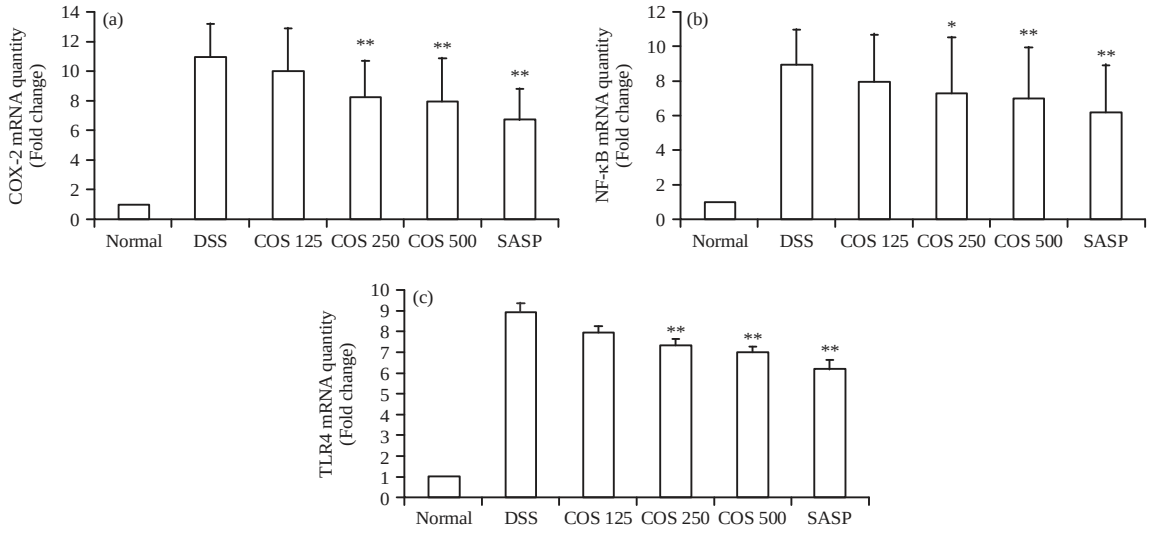

Fig. 3(a-c): Effect of COS on the mRNA expression of (a) COX-2, (b) NF-κB and (c) TLR4 in colon tissue of DSS-induced mice, *p<0.05, **p<0.01 vs DSS model group

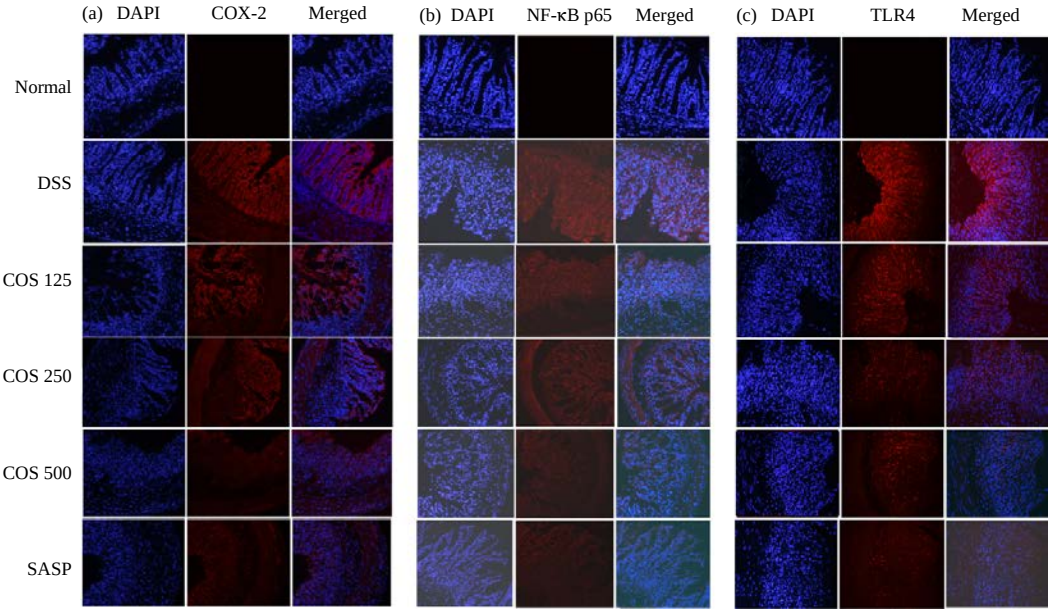

Fig. 4(a-c): Effect of COS on the protein expression of (a) COX-2, (b) NF-κB and (c) TLR4 in colon tissue of DSS-induced mice. The protein expression of COX-2, NF-κB and TLR4 was determined by immunofluorescence assay. Nuclei were stained with DAPI (4, 6-diamidino-2-phenylindole)

DISCUSSION

The anti-inflammatory medications, e.g., 5-aminosalicylic acid compounds such as salazosulfapyridine, are considered to be the first-line therapy for active UC management. However, side effects including abdominal pain, fever, diarrhea, cramps, rash and kidney failure limited their use[6]. The lack of satisfactory treatment for UC has fueled the studies exploring alternative therapeutic strategies. Anti-inflammatory natrual products or functional foods originated from complementary or alternative medicine represent a novel class of promising agents toward UC therapy[4,29]. The COS are a kind of natural products without any apparent adverse effects. And based on the fact that COS have functional properties after oral intake, bread and dairy food containing COS have been believed as the most appropriate functional

food[8,30]. The previous *in vitro* study[31] has found COS could significantly and concentration-dependently attenuated the lipopolysaccharide-induced inflammatory response in intestinal epithelial (Caco-2) cells, suggesting the potential medical use of COS in the control of inflammatory bowel diseases. The present study used an animal model of UC, a major form of inflammatory bowel disease in which the inflammation was mainly restricted to the colon by challenging the mice with DSS in drinking-water and outlined the anti-inflammatory activity of COS against experimental colitis. As expected, it was found the preventive effect of oral administration of COS on the colonic inflammation, which manifested as reduced histological damage scores and colonic PGE_2 content in COS-treated mice and further confirmed the potential of COS for experimental colitis. The effectiveness of COS appeared to close to that of SASP, a common and effective drug in the clinical treatment of UC consisting of 5-aminosalicylic acid bound to sulfapyridine via a diazo bond, which was used as a positive control in this study.

Advances in the understanding of disease mechanism for UC have greatly accelerated the discovery of many therapeutic agents targeting proinflammatory signaling[32] for example, TLR4/NF-κB/COX-2 signaling cascade. COX-2 is an inducible enzyme in the inflammatory process that contributes to the production of inflammatory mediator PGE_2. Consistent with the results about the anti-inflammatory mechanism of COS obtained in the setting of other disease[10,15,17], it was also found that COS significantly dowregulated COX-2 expression in the colonic tissue of DSS-treated m ice, which is responsible for the inhibition of subsequent PGE_2 synthesis by COS. It is well-documented that TLR4/NF-κB pathway plays a key role for the regulation of COX-2 expression in the intestine[25]. Notably, as a canonical proinflamatory signalling, TLR4/NF-κB pathway has recently been considered as an important target for UC control. Many therapeutic agents in UC that abrogate intestinal inflammation has been believed to block the TLR4/NF-κB pathway[26,28,32]. The TLR4 is induced by proinflammatory cytokines and highly expressed in inflamed mucosa of UC patients. As a pattern recognition receptor, TLR4 palys a key role in defensing against pathogens in the intestine. However, because TLR4 is considered as the most important inflammation inducer in all the TLR family members, the TLR4-mediated inflammation-associated intestinal demage further contribute to accelerate the development of UC[25-28]. The NF-κB could be activated by TLR4 stimulation and is a key transcription factor in the induction and regulation of a range of inflammatory mediators[33]. In the present study, it was found that COS treatment significantly and dose- dependently reduced the enhanced expression of TLR4 and NF-κB in the colons induced by DSS, suggesting the inhibition TLR4/NF-κB pathway together with its downstream COX-2 might be an important mechanism underlying the anti-colitic effect of COS *in vivo*.

Despite the fact that the anti-inflammatory effect of COS has been well established, the further study on its molecular mechanism is still rare. The results of this study for the first time show that TLR4 as a key molecule mediating the inflammatory activation, together with its downstream pathway are involved in the anti-inflammatory effect of COS. In view of the known role of TLR4/NF-κB/COX-2 signaling cascade in UC development, it was believed that COS ameliorated the intestinal inflammation in DSS-treated mice, at least partly by inhibiting TLR4 pathway. In fact, TLR4 signalling pathway is involved in the pathophysiology of many inflammatory diseases, it could be speculated that the downregultion of this anonical proinflamatory signalling may also be one of the mechanisms responsible for COS' protective properties in other inflammatory settings. But further studies are needed.

CONCLUSION

The present study demonstrated that oral administration of COS reduced histological damage scores of the colon and colonic PGE_2 content in DSS-treated mice, suggesting the preventive effect of COS on the colonic inflammation. Importantly, it was found that the anti-inflammatory effect shown by COS appear to be related to their capacity to down-regulate TLR4/NF-κB pathway, a canonical proinflamatory signalling, together with its downstream COX-2, indicating TLR4/NF-κB/COX-2 signaling cascade as potential target for the anti-inflammatory activity of COS in experimental colitis. These finding could give insight into the further evaluation of COS as a food supplement or an alternative agent in the control of human UC.

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