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Administration of Aqueous Extract of *Schizandra chinensis* Fruit Inhibits the Experimental Colitis in Mice

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Abstract – *Schizandra chinensis* fruits (SC) have been used as a traditional Oriental medicine for treatments of many stress-induced diseases. In the present study, we investigated the protective effect of SC aqueous extract (SC-Ex) in the inflammatory diseases of intestine using a mouse model of ulcerative colitis. An experimental colitis was induced by daily treatment with 5% dextran sulfate sodium (DSS). SC-Ex was orally administered from day 2 of DSS treatment in a dose-dependent manner. Administration of SC-Ex reduced significantly clinic signs of DSS-induced colitis, including body weight loss, shorten colon length, increased disease activity index, and histological colon injury. Moreover, SC-Ex suppressed significantly not only the activities of myeloperoxidase (MPO) and chymase, but also the expressions of TNF-á and COX-2 in DSS-treated colon tissues. Inhibitory effect of SC-Ex was effective at a dose over 20 mg/kg. Our results indicate that SC-Ex may possess therapeutic effect on the development of DSS-induced colitis.
Keywords – *Schizandra chinensis*, ulcerative colitis, colon injury, dextran sulfate sodium

Introduction

Ulcerative colitis (UC) is a typical inflammatory intestinal disease belonging to the inflammatory bowel diseases (IBD) and characterized by erosion, mucosal ulceration, and infiltration of inflammatory cells (Fiocchi, 1998; Hyams, 2000; Danese *et al.*, 2004). UC exhibits the pathological state primarily affecting the superficial layer of the colon mucosa, and UC patient's tissues show the general features, such as ulceration of mucosa, blunting and loss of crypts, and infiltration of inflammatory cells (Blumberg *et al.*, 1999). Additionally, prolonged and chronic UC is able to progress to a colorectal cancer (Shetty *et al.*, 1999). Although the conventional drugs, including aminosalicylates, corticosteroids, and immunosuppressors, have been used to UC treatment (Domenech *et al.*, 2006), they have a slow onset of action and do not respond to long-term treatment in most of patients.

Traditional Chinese herbal medicine has been increased interest for the treatment of these disorders. The fruits of *Schizandrae chinensis* (SC), a member of the Magnoliaceae family, have been used as tonic and astringent in traditional Oriental medicine (Chen *et al.*, 1993). Since SC had the capacity to increase resistance to a wide range of physical, chemical, and emotional stresses, it has been classically prescribed for the treatment of chronic cough and dyspnea, diarrhea, night sweats, wasting disorders and irritability (Sinclair, 1998). Moreover, previous studies have shown that SC has the anti-hepatotoxic and anti-neurotoxic effects through the properties of antioxidation and lipid peroxidation inhibition (Hikino *et al.*, 1984; Mak *et al.*, 1996; Ip *et al.*, 1995). The crude extracts of SC have the anti-ulcer and anti-secretory activity (Daniel *et al.*, 1998; Long *et al.*, 1985). Several studies have reported that fruits of SC comprised a variety of lignan compounds, including schizandrin, gomisin N, deoxyschizandrin, Wuweizisu C and Gomisin A, and these lignans showed protection of CCl_4-induced hepatotoxicity and glutamate-induced neurotoxicity (Ko *et al.*, 1995; Kiso *et al.*, 1985; Kim *et al.*, 2004). However, the effect of SC for protective activity on the acute and chronic colitis remains still to be studied. Scientific evidences for

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use of SC in various diseases are being investigated because of the solid clinical observations in Oriental medicine.

In the present study, we investigated the therapeutic effect of SC extract (SC-Ex) on dextran sulfate sodium (DSS)-induced experimental colitis in mice which is a useful animal model exhibiting similar phenotype to human acute and chronic UC.

Experimental

Animals and reagents – Female BALB/c mice (7 weeks old) were obtained from the SamTaco animal facility (Gyeonggi, Korea). Mice were housed in a specific pathogen-free environment for at least 1 week before experiments. DSS (mol wt; 36,000-50,000) was purchased from ICN Biomedicals (Aurora, OH), and Schizandin and gomisin N were from Chromadex INC (Santa Ana, CA). The specific antibodies against TNF-α and COX-2 were from Santa Cruz Biotechnology (Santa Cruz, CA). All chemical reagents were from Sigma Co. (St. Louis, MO).

Preparation of aqueous extracts – SC cultivated in farm of Jinan (Jeonbuk, Korea) was dried in the Wonkwang University School of Oriental Medicine (Iksan, Korea). SC (100 g/l) was decocted with distilled water for 2 h in the presence of reflux condense. The aqueous extracts of SC (SC-Ex) were filtered through 0.45 μm filter, concentrated using a rotary vacuum evaporator, and finally freeze-dried (29.5 ± 1.3 g). For oral administration, the dried extract was dissolved with distilled water and diluted appropriately with PBS. To determine the major lignan compounds in SC, the dried SC-Ex was resuspended with 90% methanol. The methanol-soluble fraction was lyophilized (24.82 ± 1.1 g) and contents of schizandin and gomisin N were analyzed by high performance liquid chromatograpy (Gilson Co.) using a proper condition [column: Sunfire™ C18 (5.0 μm), 4.6 mm × 250 mm; flow rate: 1,0 ml/min; elution solvent: acetonitrile-methanol-water (11 : 11 : 8); detector: UV 254 nm] as previously reported by K.S. Kim *et al.* Data were obtained from triplicate experiments.

Induction of colitis by DSS – Acute colitis in mice was induced by providing drinking water *ad libitum* containing 5% DSS for 7 days (Okayasu *et al.*, 1990). Mice were checked daily for loss of body weight, stool consistency and the presence of gross bleeding. Mice were randomized into groups receiving SC-Ex in dose dependent manner (4, 20 and 100 mg/kg body weight of mouse). SC-Ex diluted with saline (50 μl) was orally administrated twice a day from day 2 of DSS treatment. Aminosalisylic acid (ASA) was used as a positive control drug. Mice finally were sacrificed at day 7 after DSS treatment, and their colons were excised to assess the intestinal manifestations.

Disease activity index – The activity of intestinal disease was assessed through manifestations, comprising loss of weight, diarrhea accompanied with blood and mucus, and shortening of colon (Hendrickson *et al.*, 2002). As described by Murthy *et al.* (1993), disease activity index (DAI) was obtained from score of three major clinical signs (weight loss, diarrhea, and rectal bleeding). Loss of body weight was calculated as the difference between the initial and actual weight. Diarrhea was defined by the absence of fecal pellet formation in the colon and the presence of continuous fluid fecal material in the colon. The appearance of rectal bleeding was separated as diarrhea containing visible blood and gross rectal bleeding and scored as described for diarrhea. DAI was calculated using the following formula: DAI = (weight loss score) + (diarrhea score) + (rectal bleeding score). This method of scoring has been validated by repeated studies. A significant decrease in the DAI (*$P < 0.05$) is considered an end-point of successful therapy.

Histochemical staining – The colons were removed from mice after euthanasia. Their lengths were measured immediately and than the feces were carefully eliminated from the GI tract lumen. Colon tissues were fixed in 4% buffered paraformaldehyde (pH 7.0), embedded in paraffin, and sectioned (6 μm) onto gelatin-coated glass slides. After deparaffinization with xylene and rehydration through gradually ethanol washes (100%, 90%, 80%, 70%, and 0%), sections were stained with hematoxylin and eosin (H/E). All specimens were visualized and photographed under a microscope using camera system (Olympus LK2, Japan).

MPO activity assay – Myeloperoxidase (MPO) has been known to be a marker for the detection of neutrophil infiltration in the intestine inflammation (Krawisz *et al.*, 1984). The samples of colon were homogenized in 20 mM phosphate buffer (pH 7.4) for 30 s and centrifuged at 15,000 × g for 20 min. The pellet was re-homogenized in 10 volumes of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide (HTAB) and 10 mM EDTA on ice bath. The homogenate was then freeze-thawed once, sonicated for 1 min. and centrifuged at 15,000 × g for 20 min. The supernatant (50 μl) was mixed with 50 μl of 50 mM phosphate buffer containing 0.5% HTAB, 50 μl of *o*-dianisidine (0.68 mg/ml in distilled water), and 0.3 mM hydrogen peroxide, and than incubated for 3 min at 37°C.

The change in absorbance was measured spectrophotometrically at 450 nm. The MPO activity was standardized using pure human MPO (Sigma) and one unit of MPO level was defined as the ability able to degrade 1 μM per min at 25°C. Data were described as a relative increase of MPO activity of each group against that of normal control group.

Chymase activity assay – The distal colonic specimens were homogenized in 20 mM Na-phosphate buffer (pH 7.4) and than was centrifuged at 15,000 × g for 30 min and the supernatant, which is rich in non-chymase proteases that would interfere with the assay, was discarded and the pellets were homogenized in 5 volumes (w/v) of 10 mM Na-phosphate buffer (pH 7.4) containing 2 M KCl and 0.1% NP-40, stored overnight at 4°C, and then centrifuged at 15,000 × g for 30 min. Supernatants were used to measure the enzyme activities of chymase (Kakizoe *et al.*, 1999). The enzyme activities of chymase were determined using the substrate 0.2 mM N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide containing 50 μg/ml heparin, 1 mg/ml bovine serum albumin, 1 mg/ml aprotinin, 2 M KCl, and 100 mM Tris-HCl (pH 7.6) (Schechter *et al.*, 1988). In all assays, 50 μl of sample was added to a total reaction volume of 200 μl in a 96-well plate. This substrate is hydrolyzed by chymase and releases continuously p-nitroaniline, which is a yellow chromophore. Thereby, the multiple readings were taken and reaction rates were measured by monitoring continuously the increase in absorbance at 405 nm for 5 min using a micro-ELISA reader. The gradient of linear increase of the absorbance (A405/min) was converted into protease activity. Protein concentration was measured using a bicinchoninic acid (BCA) protein assay reagent and the chymase activity was shown as the specific activity (U/mg protein).

Western blot analysis – The distal colons (100 mg) were homogenized in 600 μl of lysis buffer (iNtRON Biotech, Korea), incubated for 30 min on ice, and centrifuged at 13,000 rpm for 5 min. The supernatants were transferred to a new tube and their protein concentrations were determined using a PRO-MEASURE solution (iNtRON Biotech). Lysates were separated by 10-15% SDS-PAGE and transferred to PVDF membrane (Amersham Pharmacia Biotech, Piscataway, NJ). After blocking with 5% skim milk, membrane was blotted with specific antibody against TNF-α, COX-2 or β-actin for 18 h at 4°C. After washing in 1 × TBS containing 0.1% Tween 20, the immunobloted membrane was incubated with the goat anti-mouse IgG-horseradish peroxidase (HRP) conjugate at a 1 : 10,000 dilution for 45 min. Finally, the epitopes on proteins recognized by the specific antibody were visualized by using the enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

Statistical analysis – All values from *in vivo* experiments (n = 5) were described to mean ± S.E.M. Statistical significance was determined using the Student's *t*-test to express the difference between groups. All p-values < 0.05 were considered to reflect a statistically significant difference.

Results

SC-Ex reduces the clinical signs in DSS-induced colitis – We examined the inhibitory effects of SC-Ex on the intestines in DSS-induced experimental colitis. As shown in our previous study (Choi *et al.*, 2005), the histological and physiological signs (weight loss, colon length, diarrhea, and occult/gross bleeding) were observed

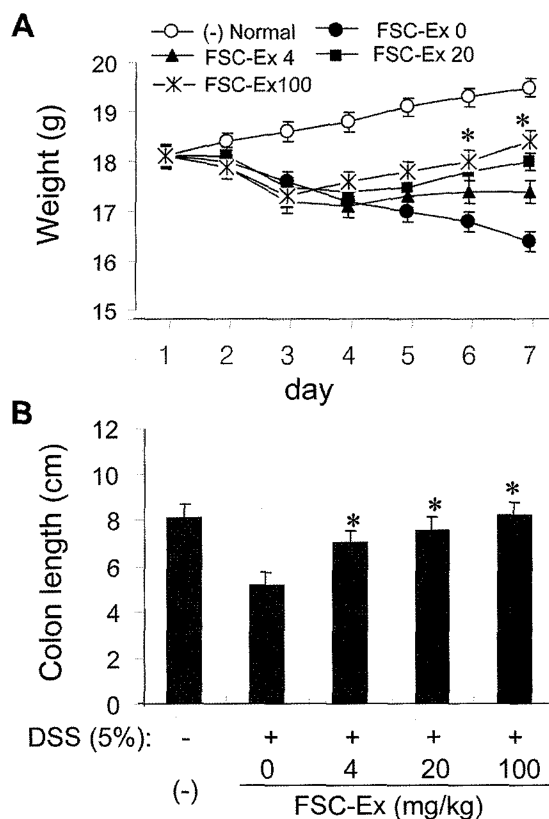

Fig. 1. Effect of SC-Ex on the wasting of weight and colon length in DSS-induced colitis. Colitis was induced by oral administration of DSS (5%) for 7 d. At day 2 of DSS treatment, SC-Ex was administrated orally at various doses (4, 20 and 100 mg/kg weight). (A) Body weight of mice was measured daily after DSS treatment. (B) The colons were removed at day 7 after DSS treatment, and the colon lengths were measured. Data were represented in the mean ± S.E.M. (n = 5) from triplicate experiments (*p < 0.05 vs. DSS alone).

separately by 5% DSS treatment for 7 d, and their DAIs were calculated. All mice treated with DSS alone for 7 d showed significant weight loss and colon shortening compared to normal control group. SC-Ex was orally administrated twice a day at the different three doses (4, 20 and 100 mg/kg weight). We observed that groups administrated with SC-Ex showed the significant attenuation of body weight loss (Fig. 1A) and colon shortening (Fig. 1B) caused by DSS treatment in dose dependent manner. However, SC-Ex alone without DSS treatment did not exhibit undesirable damage. In addition, DAI was inhibited remarkably in groups administrated with SC-Ex compared to group of DDS alone (Fig. 2). These results indicate that SC-Ex might inhibit effectively the symptoms of colitis caused by DSS.

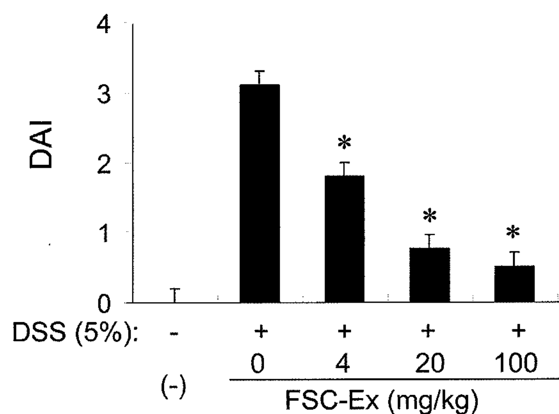

Fig. 2. Effect of SC-Ex on DAI in DSS-induced colitis. Colitis was induced by oral administration of DSS (5%) for 7 d. At day 2 of DSS treatment, SC-Ex was administrated orally at various doses (4, 20 and 100 mg/kg). DAI was calculated as described in Materials and Methods. Data were represented in the mean ± S.E.M. (n = 5) from triplicate experiments (*$p < 0.05$ vs. DSS alone).

SC-Ex reduces histological damages in colon tissue – Next, we evaluated the inhibitory effect of SC-Ex on the histological damages of colon resultant from DSS treatment. As shown in Fig. 3B, there was a typical lesion of colon in DSS-treated group manifested by multifocal areas, mucosal erosion, a loss of epithelial and goblet cells, the shortening and collapse of crypts, and submucosal edema. At day 7 after DSS treatment, the distal colon tissues were cut and fixed. Sections were stained by hematoxylin and eosin. Administration of SC-Ex (4, 20 and 100 mg/kg) reduced remarkably lesions of colon in DSS-induced colitis in dose-dependent manner (Fig 3C, D, and E). Especially, the protective and healing effects of SC-Ex on the colon damages were more prominent at the higher dose, and the inhibitory effect of over 20 mg/kg SC-Ex was comparable to that of group administrated

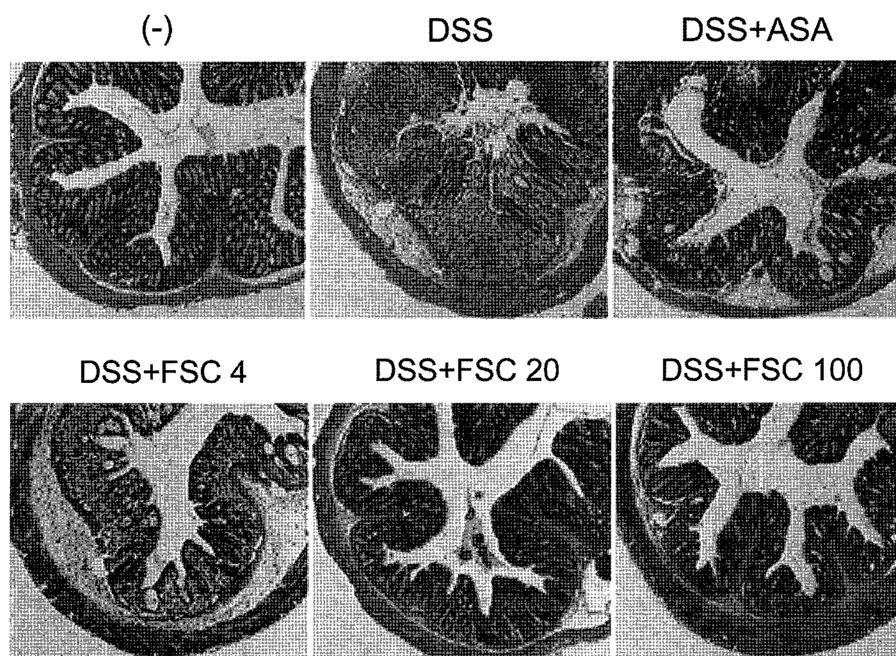

Fig. 3. Effect of SC-Ex on histological damage of colon tissues in DSS-induced colitis. The colon tissues treated with DSS for 7 d showed the histological change (such as crypt loss, surface epithelial loss, and infiltration of inflammatory cells into the mucosa). At day 2 of DSS treatment, SC-Ex was administrated orally at various doses (4, 20 and 100 mg/kg), and ASA (50 µg/kg) was used as a comparative drug. Colon specimens were paraffin embedded and stained with hematoxylin and eosin (magnification, ×100). The histological damage of the colon wall structure by DSS treatment was reduced prominently by SC-Ex administration.

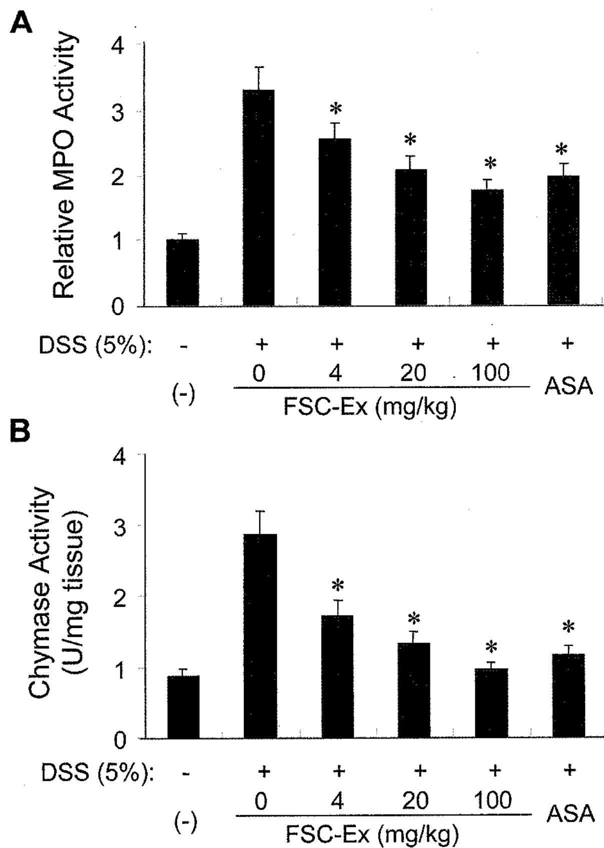

Fig. 4. Effect of SC-Ex on the activity of MPO and chymase in DSS-induced colitis. At day 2 of DSS treatment, SC-Ex was administrated orally at various doses (0, 4, 20 and 100 mg/kg), and ASA (50 μg/kg) was used as a comparative drug. The activities of MPO (A) and chymase (B) in colon tissues were determined at day 7 after DSS treatment as described in Materials and Methods. Data were represented in the mean ± S.E.M. (n = 5) from triplicate experiments (*p < 0.05 vs. DSS alone).

with ASA (50 μg/kg), a well-known drug for UC treatment. These results indicate that SC-Ex might inhibit effectively the inflammation-related damage of colon in this model.

SC-Ex reduces the activity of inflammatory enzymes in colon tissue – The intestinal inflammation is involved in the infiltration of inflammatory cells. In particular, neutrophil-derived MPO has remarkably increased activity in intestinal tissue of DSS-treated mice (Krawisz *et al.*, 1984). Therefore, we examined MPO activity in colonic homogenates consequent upon SC-Ex administration. SC-Ex reduced significantly the MPO activity elevated by DSS treatment in dose-dependent manner. In addition, the attenuation of MPO activity at the higher dose of SC-Ex (over 20 mg/kg) was comparable to that of group administrated with ASA (Fig. 4A). On the other hand, we examined the activity of mast cell-mediated chymase by SC-Ex administration. In the previous study, we have shown that mast cells were involved in DSS-induced

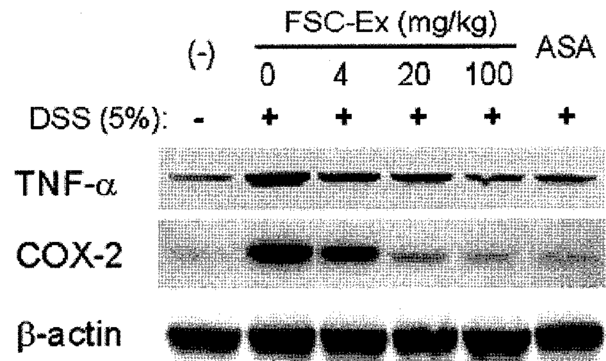

Fig. 5. Effect of SC-Ex on the expression of TNF-α and COX-2 in DSS-induced colitis. At day 2 of DSS treatment, SC-Ex was administrated orally at various doses (0, 4, 20 and 100 mg/kg), and ASA (50 μg/kg) was used as a comparative drug. The analysis of TNF-α and COX-2 protein was performed at day 7 after DSS treatment by western blot analysis described in the Materials and Methods.

colitis (Kang *et al.*, 2006). We also observed that the increased chymase activity in DSS-treated group significantly by SC-Ex administration in dose-dependent manner (Fig. 4B). These results strongly support that SC-Ex inhibited the infiltration and activation of inflammatory cells in this model.

SC-Ex inhibits the expression of inflammation inducing factors in DSS-induced colitis – We next examined the inhibitory effects of SC-Ex on the expression of inflammation-initiating factors, TNF-α and COX-2, in the tissues of colitis. After DSS treatment for 7 d, the colon tissues were cut out and homogenized. The expression levels of TNF-α and COX-2 were evaluated by western blot analysis using their specific antibodies. The expressions of TNF-α and COX-2 were significantly increased in the colon tissues of DSS-treated mice compared to that of control. We observed that oral administration of SC–Ex reduced significantly the expression of TNF-α and COX-2 induced in DSS-treated colon tissues (Fig. 5). Especially, the inhibitory effect of SC-Ex was dependent on the dose administrated. These results implicate that SC-Ex could attenuate the inflammatory responses in DSS-induced colitis.

Discussion

In the present study, we demonstrated that SC-Ex has the inhibitory effects on the inflammatory response and colon injury provoked by DSS treatment. In addition, although the dried fruits of Schizandra have been known to attenuate intestinal diarrhea in Korea, our study is the first *in vivo* report for the evaluation of the pharmaco-

logical effects of SC.

The current studies have reported that the production of pro-inflammatory cytokines was involved in the initiation of the inflammatory response in colitis. In particular, the expression levels of TNF-α (D'Haens, 2003) and IL-8 were elevated remarkably in IBD patients (Ajuebor *et al.*, 2002; Banks *et al.*, 2003). The stimulation of intestinal epithelial cells with TNF-α induced the production of IL-8, a major chemokine responsible for chemoattraction of neutrophils (Papadakis, 2004). Additionally, DSS elicits COX-2 expression to regulate the production of prostaglandins, which induce gastrointestinal fluid secretion on the inflammatory pathway (Kim *et al.*, 1998). Sulfasalazine and mesalazine (ASA) being commonly used to UC treatment have been reported to inhibit TNF-α-stimulated IL-8 expression in young adult mouse colon cells (Dijkstra *et al.*, 2002). In the present study, our results also showed that SC-Ex attenuates significantly level of TNF-α and recruitment of neutrophils in the colon tissues, and blockade of and mast cell activation and subsequently also attenuate the activity of MPO and chymase, respectively, in DSS-stimulated colon tissue.

Schizandra has been used in the Orient as an astringent. Recent studies have shown that SC comprises enriched dibenzocyclooctadiene lignans (Nakajima *et al.*, 1983), and that these lignans significantly attenuate the L-glutamate-induced neurotoxicity (Kim *et al.*, 2004; Kim *et al.*, 2002) and CCl_4-induced hepatotoxicity (Ko *et al.*, 1995) through the inhibition in the increase of intracellular [Ca(2+)] and the formation of cellular peroxide. DSS used in our study also has been known to induce oxidative intestinal damage (Oh *et al.*, 2006). We thus considered the protective effect of Schizandra fruits on the DSS-induced colon damage. Our data showed that oral administration of SC-Ex significantly reduced the clinical symptoms and the histological change of colon tissues in DSS-treated mice. We also found that SC-Ex contained schizandrin and gomisin N, which are major lignan compounds. Contents of schizandrin and gomisin N in SC-Ex revealed 0.13 ± 0.02 and 0.11 ± 0.02, respectively (data not shown), indicating that lignan compounds from Schizandra may possess therapeutic effect to inflammatory intestine damage induced by stress. Moreover, the activated inflammatory cells, including neutrophils, mast cells and macrophages, are major components of the intestinal lesions in ulcerative colitis (Suematsu *et al.*, 1987; Shiratora *et al.*, 1989; Hirata *et al.*, 2001). However, although cellular mechanism that FCS-Ex led to suppression of DSS-induced colitis is not clearly understood, it is possible that antioxidative activity of these lignans contributes to in the pathogenesis of DSS-induced ulcerative colitis. Natural products that attenuate the influx of Ca^{2+} and further improve the antioxidative defense system might offer a useful therapeutic choice in the treatment of degenerative disorders caused by oxidative stress (Simmonds *et al.*, 1993).

In conclusion, we demonstrated at first that the treatment of SC-Ex could reduce significantly the clinical signs and the expression of inflammatory mediators in a colitis model caused by DSS treatment. Thereby, our data suggest that the SC-Ex may be a useful therapeutic candidate for ulcerative colitis. However, the further studies must be performed to elucidate the precise mechanism of SC-Ex for the treatment of intestinal inflammatory disorders.

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