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The improving effects of Saengmaeksan on ulcerative colitis

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SUMMARY

Saengmaeksan (SMS) is a well known Korean traditional herbal prescription, which consists of 3 different herbs, *Radix Ophiopogonis, Radix Ginseng, and Fructus Schisandrae*. SMS has been commonly used in various disease including respiratory system and cardiovascular System in Korea. The objective of this study is to find out the improving effects of SMS against Dextran Sulfate Sodium (DSS)-induced ulcerative colitis. SMS reduced clinical signs of DSS-induced colitis, including body weight loss, shorten colon length, and increased disease activity index. The results showed that SMS significantly inhibited the activation of nuclear factor-kB p65 in the colon tissues of DSS-treated mice. In addition, we observed that result showed that the levels of IL-6 in plasma were increased in DSS treated group compared to those of the normal group, but these increased levels were reduced by administration with SMS. Taken together, these findings suggest that SMS has improving effects on DSS-induced ulcerative colitis**,** which may explain its beneficial effect in the regulation of chronic intestinal inflammation**.**

Key words: Saengmaeksan; Dextran Sulfate Sodium; Ulcerative colitis; Nuclear factor-κB p65; Interleukin-6

INTROUDCTION

Ulcerative colitis (UC) is a typical inflammatory intestinal disease belonging to the inflammatory bowel diseases (IBD) (Fiocchi, 1998; Hyams, 2000). The pathogenesis of UC is believed to result from the interaction of genetic, immune, and environmental factors (Danese *et al.*, 2004). UC is associated with intestinal and often results in weight loss, diarrhea accompanied with blood and mucus, fever, gastric dysmotility, and shortening of the colon (Hendrickson *et al.*, 2002). General features of UC tissue are ulceration of the mucosa, blunting and loss of crypts, and infiltration of inflammatory cells (Blumberg *et al.*, 1999). These conditions frequently cause epithelial dysplasia and DNA damage with microsatellite

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instability, and eventually can progress to cancer (Ullman *et al.,* 2003). Indeed, involvement of the entire colon for longer than 10 years predisposes UC patients to colon cancer (Ullman *et al.,* 2003), indicating the essential contribution of chronic inflammation to colon carcinogenesis. Thus, to develop measures to prevent cancer development in UC patients, it is necessary to gain an understanding of the pathogenesis of UC at molecular and cellular levels.

Current studies on UC have reported that pro-inflammatory cytokines are involved in the initiation of the inflammatory response in colitis. In particular, the level of IL-6 has been found to be remarkably elevated in UC patients (Li *et al.,* 2010).

Nuclear factor (NF)-κB is one of the most important transcription factors for the induction of genes mediating innate and adaptive immunity. The prototype of NF-κB is a heterodimer consisting of p50 and p65 bound by members of the IκB family, including IkB-α, in the cytoplasm (Barnes and Karin, 1997; Schaecher *et al.,* 2004). Phosphorylation of IκB by bacterial products, viruses, drugs, and cytokines rapidly leads to IκB degradation and translocation of NF-κB to the nucleus. Activation of NF-κB results in the binding of specific promoter elements and the expression of mRNAs for proinflammatory cytokine genes (Ghosh *et al.,* 1998). NF-κB p65 has been shown to be critically important in chronic inflammatory diseases. Inhibition of NF-κB activation has been suggested as an anti-inflammatory strategy in IBD (Neurath *et al.,* 1998). Generally, UC is associated with intervals of acute exacerbation, and the administration of corticosteroids is effective in bringing about a clinical remission (Domenech, 2006). However, in some severely relapsed cases, corticosteroids are not always effective even when a high dosage of more than 1 mg/kg/day is administered orally or intravenously. In addition, the long-term use of corticosteroids often causes serious side effects such as hormonal disturbance, peptic ulcers, liver dysfunction and psychological problems. These problems sometimes cause the disruption of corticosteroid treatment and result in acute exacerbation. Therefore, an alternative treatment for active UC is necessary in order to avoid these clinical problems associated with corticosteroid therapy. Traditional herbal medicine has seen increased interest for the treatment of these disorders. Saengmaeksan (SMS) is a well known Korean traditional herbal prescription, which consists of 3 different herbs, *Radix Ophiopogonis, Radix Ginseng, and Fructus Schisandrae*. SMS has been used in traditional Korean medication for pyrogenic disease. However, the improving effects of SMS on chronic intestinal inflammation are poorly understood.

Another study reported that dextran sodium sulfate (DSS)-induced colitis in mice has a phenotype similar to human acute and chronic UC (Okayasu *et al.,* 1990). The aim of this study is to examine the the improving effects of SMS on the pathogenesis of DSS-induced colitis. The specific aims were as follows: To assay the effect of SMS on clinical signs including weight loss, colon length, diarrhea, and occult/gross bleeding; to investigate the effect of SMS on inflammatory-related gene expression in DSS-treated colon tissues.

MATERIALS AND METHODS

Animals and reagents

Female BALB/c mice (6 weeks old) were obtained from the Da-Mool Science (Taejeon, Korea). Mice were housed in a specific pathogen-free environment for at least 1 week for adaptation to the environmental changes and were sacrificed by CO_2 inhalation at the end of the study. DSS (mol wt; 36,000 - 50,000) was purchased from MP Biomedicals (Solon OH). Purified anti-mouse IL-6 and recombinant IL-6 were obtained from BD-Pharmingen (San Diego, CA). The specific antibodies against COX-2 and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). All chemical reagents were from Sigma Co. (St. Louis, MO).

Table 1. The Ratio of the Component in SMS

Component of SMS	Ratio
1. *Radix Ophiopogonis*	8 g
2. *Radix Ginseng*	4 g
3. *Fructus Schisandrae*	4 g

Preparation of SMS

The SMS which is a mixture of three traditional drugs as shown in Table 1 was purchased from Daehak Oriental Pharmacy (Iksan, Korea). Extract of SMS was prepared by decocting the dried prescription with boiling distilled water. The extraction decocted for approximately 3 h was filtered, lyophilized, and kept at 4°C. The yield of dried extract from starting materials was about 3%. Dilutions were made in saline and filtered through 0.22 um syringe filter.

Induction of colitis by DSS

Acute colitis in mice was induced by providing drinking water ad libitum containing 5% (w/v) DSS for 7 days. Mice were checked daily for loss of body weight, stool consistency and the presence of gross bleeding. Mice were randomized into groups receiving SMS (1 g/kg), sulfasalazine (150 mg/kg) as a positive control, or water as a negative control. SMS and sulfasalazine diluted with water (200 ml) were orally administrated once a day from day 0 of DSS treatment. The mice were finally sacrificed and assessed after 7 days of DSS treatment.

Disease activity index (DAI)

The activity of intestinal disease was assessed through manifestations comprising loss of weight, diarrhea accompanied with blood and mucus, and shortening of the colon (Hendrickson *et al.*, 2002). As described by Murthy *et al.* (1993), a disease activity index (DAI) was obtained from the 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). The clinical parameters used here are comprehensive functional measures that are analogous to the subjective clinical symptoms observed in human ulcerative colitis. This method of scoring has been validated by repeated studies.

Cytokine assay

Blood samples were obtained from mouse and immediately centrifuged at 3,000 rpm for 15 min to separate plasma. The levels of IL-6 secretion were measured using a modification of the enzyme-linked immunosorbent assay (ELISA) described elsewhere (Jeong *et al.*, 2003). 96 well plates were coated with 100 ml aliquot of anti-mouse IL-6 monoclonal Abs at 1.0 mg/ml in PBS at pH 7.4 and incubated overnight at 4°C. Additional washes, 100 ml of the sample or the IL-6 standards were added and incubated at 37°C for 2 h. After 2 h incubation at 37°C, the wells were washed and then 0.2 ug/ml of biotinylated anti-mouse IL-6 was then added and again incubated at 37°C for 2 h. After washing the wells, avidin-peroxidase was added and plates were incubated for 30 min at 37°C. The wells were washed again and ABTS substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using the recombinant IL-6 in serial dilutions. Protein concentration was measured using a bicinchoninic acid (BCA) protein assay reagent (Sigma).

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 fresh tube and their protein concentrations were determined using BCA protein assay reagent (Sigma). Lysates (50 ug of protein) were separated by 10% SDS-PAGE and transferred to membranes (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was then blocked with 5% skim milk in phosphate-buffered saline (PBS)-tween-20 for 1 h at room temperature and then incubated with the antibodies. After washing in PBS-tween-20 three times, the blot was incubated with the secondary antibody for 1 h and the antibody-specific proteins were visualized using an enhanced chemiluminesence detection system according to the recommended procedure (Amersham Corp. Newark, NJ, USA).

Statistical analysis

The results are presented as the mean ± S.E.M. of at least three experiments. The results were examined using independent *t*-tests and ANOVA with a Tukey *post hoc* test. A P value of < 0.05 was considered significant.

RESULT

The effect of SMS on clinical signs in DSS-induced colitis

The inhibitory effects of SMS on the intestines in DSS-induced experimental colitis were evaluated from the experiment. The physiological signs (weight loss, colon length, diarrhea, and occult/ gross bleeding) were observed by 5% DSS treatment for 7 days, and their DAIs were calculated. As shown in Fig. 1A and B, all mice treated with DSS showed significant weight loss and colon shortening compared to the control group. However, we observed that groups administrated with SMS showed significant attenuation of the body weight

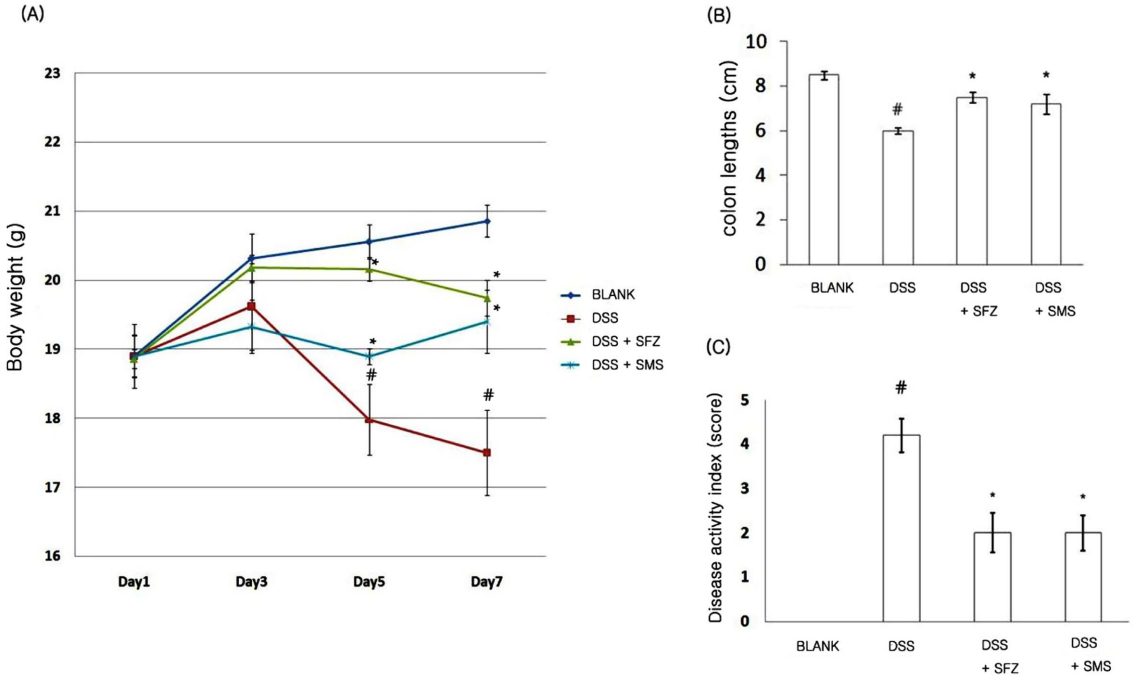

Fig. 1. Effect of SMS on clinical signs in DSS-induced colitis. Experimental colitis in mice was induced by a 5% DSS dissolved in the drinking water for 7 days. SMS was administered orally at doses of 1 g/kg once a day for 7 days prior to 5% DSS supplement. (A) Body weight of mice was measured. (B) The colons were removed at day 7 after DSS treatment, and the colon lengths were measured. (C) Disease activity index = was calculated. Sulfasalazine (150 mg/kg) was used as a positive control. Data were represented in the mean ± S.E.M. (n = 5) from triplicate experiments ($^{\#}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. DSS alone).

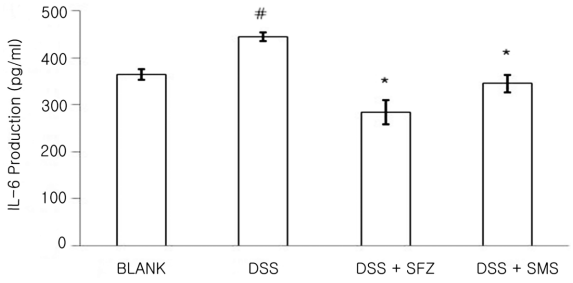

Fig. 2. Effect of SMS on the level of IL-6 in DSS-treated mouse plasma. Experimental colitis in mice was induced by a 5% DSS dissolved in the drinking water for 7 days. SMS was administered orally at doses of 1 g/kg once a day for 7 days prior to 5% DSS supplement. Sulfasalazine (150 mg/kg) was used as positive control. At the end of experiment, blood samples were obtained from mouse and immediately centrifuged at 3,000 rpm for 15 min to separate plasma. The levels of IL-6 in mouse plasma were evaluated by ELISA.

loss and colon shortening caused by DSS. In addition, DAI was remarkably inhibited in groups administrated with SMS as compared to the DSS group (Fig. 1C). As sulfasalazine has been used as a positive control in the present study.

The effect of SMS on levels of IL-6 in DSS-induced colitis

To investigate the effect of SMS on IL-6 levels in mouse plasma affected by colitis, ELISA was performed. At the end of the experiment, blood samples were obtained from mouse. As shown in Fig. 2, the levels of IL-6 were significantly increased in DSS-treatment group compared to those of the control group. However, administration of SMS reduced these increased levels induced by DSS.

The effect of SMS on expression of NF-κB p65 in DSS-induced colitis

Activation of NF-κB p65 is involved in colitis, and thus inhibition of NF-κB activation has been suggested as an anti-inflammatory strategy in colitis (Neurath *et al.,* 1996). We examined whether SMS regulated the activation of NF-κB p65 in the tissues affected by colitis. The activation of NF-κB p65 was significantly increased in the colon tissues of DSS-treated mice compared to those of the control group. Oral administration of SMS significantly reduced the activation of NF-κB p65 induced in DSS-treated colon tissues (Fig. 3).

DISCUSSION

In the present study, we investigated the improving effects of SMS on DSS-induced experimental colitis in mice which is a useful animal model exhibiting similar phenotype to human acute and chronic UC.

Ulcerative colitis (UC) is a non-specific inflammatory intestinal disease. The pathogenesis of UC is affected by a variety of factors, but its pathogenesis is still unknown at present (Heng *et al.,* 2005). Although corticosteroids are highly effective and widely used to induce clinical remission in patients with chronic intestinal inflammation,

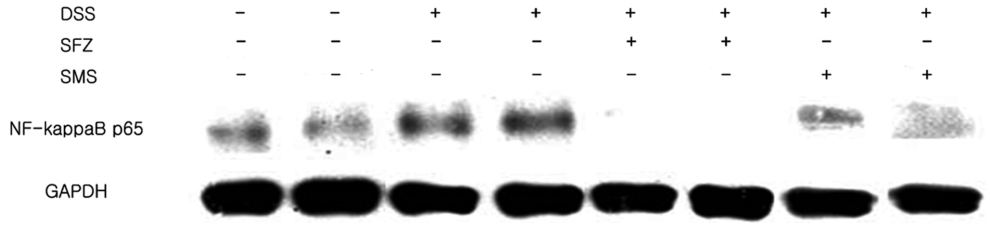

Fig. 3. Effect of SMS on the level of NF-κB p65 in DSS-treated colon tissue. Experimental colitis in mice was induced by a 5% DSS dissolved in the drinking water for 7 days. SMS was administered orally at doses of 1 g/kg once a day for 7 days prior to 5% DSS supplement. Sulfasalazine (150 mg/kg) was used as positive control. At the end of experiment, the colon tissues were cut out and homogenized. The levels of NF-κB p65 were evaluated by Western blot analysis.

chronic use of corticosteroids should be avoided because of the risk of dependency and relapse, and to prevent the development of serious and sometimes irreversible adverse effects. Recently, traditional herbal medicine has seen increased interest for the treatment of these disorders.

In the present study we examined the improving effects of SMS which is a well known Korean traditional herbal prescription on the pathogenesis of colitis. SMS has been commonly used in various disease including respiratory system and cardiovascular system in Korea. Also, it has been used for the treatment of loss of essence-energy and excessive body fluid, and is especially prescribed for coronary artery disease (Lee and Yadav, 2006). Various studies have evidenced the effectiveness of SMS in the management of immune or inflammatory responses. For instance, SMS has been shown to protective effect on endotoxin induced systemic inflammatory reaction syndrome (Wei *et al.,* 1996). In addition, SMS has been shown to protective effect on cardiac function and inflammatory reaction in patients with acute coronary syndrome (Zhang *et al.,* 2008). However, the anti-colitis effect of SMS is not understood. UC is a chronic intestinal inflammation resulting in symptoms that include abdominal pain, weight loss and bloody diarrhea (Ardizzone and Bianchi, 2005; Rufo and Bousvaros, 2006; Sato *et al.,* 2007).

In this study, we observed that mice treated with DSS showed weight loss and colon shortening compared to a control group. However, treatment with SMS reduced the weight loss and colon shortening caused by DSS. DAI, which was scored with three major clinical signs (weight loss, diarrhea, and rectal bleeding) was remarkably inhibited in groups administrated with SMS compared to DSS group not treated with SMS. These results suggest that SMS might effectively inhibit the symptoms of colitis caused by DSS.

The development of new biological therapies for UC has focused on blocking the inflammatory cascade including cytokines. Pro-inflammatory cytokines such as IL-6 are involved in the initiation of the inflammatory response in colitis. In particular, the level of IL-6 is remarkably elevated in UC patients (Li *et al.,* 2010). In this study, we showed that the levels of IL-6 in plasma were increased in DSS treated group compared to those of the normal group, but these inductions were reduced by treatment with SMS. These results indicated that the anti-inflammatory activity of SMS is due to regulation of inflammatory mediators in DSS-induced colitis.

NF-κB is a transcription factor that is important for the activation of many inflammatory mediators such as cytokines. NF-κB p65 has been shown to be critically important in chronic inflammatory diseases. Inhibition of NF-κB activation has been suggested as an anti-inflammatory strategy in IBD (Neurath *et al.,* 1996). In this study, we observed that the activation of NF-κB p65 was significantly increased in the colon tissues of DSS-treated mice compared to those of the control group. However, SMS reduced this induction in DSS-treated colon tissues. Our results provide a novel mechanism by which SMS regulates NF-κB p65 activation in colitis caused by DSS.

In summary, we have demonstrated that a treatment of SMS can significantly reduce the clinical signs and the levels of inflammatory mediators in a colitis model caused by DSS treatment. Based on these results, we suggest that SMS may be a useful therapeutic candidate for colitis. However, the further studies must be performed to elucidate the precise mechanism of SMS for the treatment of intestinal inflammatory disorders.

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