韓藥作誌(Korean J. Medicinal Crop Sci.) 19(1) : 16 – 23 (2011)

매실 혼합물이 DSS로 유도된 염증성 장질환 동물모델의 면역조절에 미치는 활성

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Anti-Inflammatory Effects of *Prunus mume* Mixture in Colitis Induced by Dextran Sodium Sulfate

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ABSTRACT : This study was conducted to investigate the anti-inflammatory effects of *Pruns mume, Schisandra chinensis, Chaenomeles sinensis-- Prunus mume* mixtrue (PM) treatment on colitis induced in mice by dextran sodium sulfate (DSS) treatment. A total of 25 male BALB/c mice (average weight 20.7 ± 1.6 g) were divided into 5 treatment groups and fed a commercial diet (A), PM administration (B), commercial diet + induced colitis by DSS (C), PM administration + induced colitis by DSS (D) and sulfasalazine + induced colitis by DSS (E). We found that PM treatment (D) and sulfasalazine (E) decreased the expression of TNF-α and COX-2 compared to the DSS-induced colitis group (C). The expression of IL-4, STAT6, IFN-γ, STAT1 was decreased in group D and group E compared to the colitis group (C), COX-2 and STAT1 were more decreased in group D. The serum IgE levels decreased in the PM treatment groups (C and D) compared to the non-PM treatment groups (A and B) although there was no significant difference between the PM treatment groups. It is notable that a therapeutic application of the PM extracts ameliorated DSS-induced colitis in mice.

Key Words **:** Dextran Sodium Sulfate (DSS), *Prunus mume*, TNF-α, COX-2

INTRODUCTION

Inflammatory bowel disease (IBD) are idiopathic chronic, relapsing intestinal disorders of complex pathogenesis, which are represented mainly by Crohn's disease (CD) and ulcerative colitis (UC) (Ghia *et al*., 2009). Patients with IBD suffer from abdominal pain and cramps, weight loss, diarrhea, disrupted digestion, rectal bleeding, and a substantial burden on everyday life (Anneke Rijnierse *et al*., 2007). IBDs are common multi-factorial intestinal disorders leading to chronic inflammation, destruction of intestinal mucosa, and the manifestation of clinical symptoms (including abdominal pain, vomiting, diarrhea and weight loss) (Levine and Fiocchi, 2000). IBDs are generally determined by deregulation of the mucosal immune response toward luminal gut bacteria antigens and by autoimmune events, such as elevated production of pro-inflammatory cytokines and increased activation of immune cells (Sellon *et al.,* 1998; Werner and Haller, 2007). The etiology of IBD remains largely undefined, but both genetic predisposition and environmental exposure are thought to contribute to the initiation of the pathogenesis (Hampe *et al.,* 2000; Schreiber and Hampe, 2000).

Prunus mume is one type of fruit tree that belongs to the Rosaceae family. From ancient times, the fruit of *P. mume* (*Ume*) has been appreciated for its ediblity and medicinal use as pickled *P. mume* (*Ume-boshi*), liqueur (*Ume-shu*), or fruit juice concentrate (*Ume* extract). Previously, fruits of *P. mume* and their processed products have been revealed to be rich in bioactive compounds, such as anti-cancer substances (Jeong *et al.,* 2006; Adachi *et al.,*

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Received 2010 July 30 / 1st Revised 2011 December 21 / 2nd Revised 2011 February 10 / Accepted 2011 February 11

2007), anti-oxidants (Shirasaka *et al.,* 1999; Jo *et al.,* 2006), and mumefural (Chuda *et al.,* 1999). Kernels of seeds also possess biological activities, such as mitogenesis (Dogasaki *et al.,* 1996).

Schisandra chinensis is generally used as a tonic medicine in Korea and it is a medical plant used in Chinese medicine. Infusions, decoctions, tinctures, and extracts from different parts of *schisandra* include large percentages of ether oils, resins, trace amounts of vitamin C, tannins, and staining materials, and large amounts of lipid soluble compounds. Moreover, *Schisandra chinensis* fruits contain a variety of pharmacologically active lignans. These compounds have various pharmacological activities, including detoxificant, anti-oxidant, anti-carcinogenic, anti-hepatotoxic, and anti-inflammatory activities (Panossin *et al*., 2008).

The fruit of *Chaenomeles sinensis*, one of the best sources, is available from plants which are widely distributed throughout China and Japan. In China, where it is known as Mu Gua, it is used as a traditional Chinese medicine to treat throat diseases; it has been used in numerous Chinese health drinks. In Japan, it is processed as a traditional fruit liquor, and is ususlly added to lozenges to treat sore throats and coughs (Reiko Sawai *et al*., 2008).

Thus, the purpose of this study was to evaluate the ability of PM mixture to inhibit IBD, especially colitis in the mice model.

MATERIAL AND METHODS

1. Materials

Sulfasalazine, dextran sulfate sodium (DSS) was purchased from Sigma (St. Louis, MO, USA). Monoclonal antibodies and cytokines were purchased from ID Labs Inc. (Ontario, Canada). IgA related antibodies were purchased from Zymed Laboratories, Inc. (San Francisco, CA, USA). IgE related antibodies were purchased from Biosource International (Camarillo, CA, USA). Dulbecco's Modified Eagle's medium (DMEM), and 3-(4,5 dimethythiazol2-yl)-2,5-diphenytetrazoleum (MTT) were obtained from Wako Chemical Co. (Tokyo, Japan). Fetal bovine serum (FBS) and antibiotics were purchased from Gibco-BRL (Gaithersburg, MD, USA).

2. Methods

1) The preparation of *Prunus mume* mixture

The *Prunus mume, Schizandra chinensis, Chaenomelis fructus* and *Prunus mume* mixture (PM) were ground to a fine powder with a grinder. The powder was extracted at 85℃ with water for 3 hr for a total of 3 extractions. The residue was extracted at room temperature and filtered again. The extract was dried by a rotary evaporator under vacuum at 40℃ and freeze dried at –70 ℃ and then stored at –20℃ until use. When after in vitro test, we decided the mixture ratio-- *Prunus mume: Schizandra chinensis: Chaenomelis fructus* = 5 : 3 : 2 (DATA not shown). PM extracts were dissolved in water and used for the animal experiments.

2) Animal treatment

A total of 25 Male BALB/c mice (average weight 20.7 ± 1.6 g) were obtained from Orient Bio (Seongnam, Korea). Animals were acclimatized under controlled conditions for 1 week before experimental feeding, and animals were housed in wire-bottomed individual cages in a windowless room on a 12-h light/dark cycle, under a protocol approved by the Institutional Animal Care and Use Committee of KonKuk University. Diet and sterilized water were provided *ad libitum* throughout the experiment. After 1 week of adaptation, 25 animals were randomly divided into 5 groups. Group A was fed a commercial diet, and group B was fed a commercial diet with colitis induced by DSS. Group C was administrated PM (50 ㎎/㎏), group D was administrated PM (50 ㎎/㎏) with colitis induced by DSS, and group E was administrated sulfasalazine (3 ㎎/㎏) with colitis induced by DSS (Ye and Lim, 2010). After 7 days of DSS administration, the mice resumed drinking plain water, and mice were administered oral doses of PM (50 ㎎/㎏) for 15 days.

3) Induction of colitis

Colitis was induced by DSS administration as previously described by Cooper *et al*. (1993). Briefly, acute colitis was induced by feeding the mice with a 3% aqueous solution of DSS over 7 days. After 7 days of DSS administration, the mice were treated with plain water for 15 days.

4) Enzyme-linked immunosorbent assay of mice antibodies

Antibodies IgE and IgA were measured by using sandwich ELISA methods, as reported previously by Lim *et al*. (2005).

5) Flow cytometry analysis

Splenocytes were obtained from the mice through the mechanical dissociation of the spleen and MLN by lysis of the red blood cells. The surface molecules of T cells were observed in spleens by the mAb directed to CD4, CD8 and a control isotope conjugated with fluorochromes (FITC, PE). All antibodies were purchased from BD Pharmingen (San Diego, CA, USA),

and sample analyses were carried out on a FACS Vantage flow cytometer (BD Bioscience, San Jose, CA, USA). Absolute cell numbers were calculated by multiplying the flow cytometry percentage by the total numbers of viable cells.

6) Histological preparation

After administered oral doses of PM (50 ㎎/㎏) for 15 days, we killed the mice and took colon. Colon tissue samples approximately 3*5 ㎜ in size were cut using a scalpel and placed in an Eppendorf vial containing 5 ㎖ of 4% parafornaldehyde in phosphate buffered saline (PBS) for overnight fixation. Samples were blotted on dry filter paper to remove excess sucrose solution before embedding in Tissue-Tek O.C.T. compound. Cryostat-section, 10 ㎛ thickness and then stained with hematoxylin-eosin for immediate histological diagnosis.

7) Western blot analysis

Cellular proteins were extracted from the mice with DSS-induced colitis. The cells were collected by centrifugation and washed once with phosphate buffered saline (PBS). The washed cell pellets were suspended in an extraction lysis buffer (Pierce) and incubated for 10 min at 4℃. The cell debris was removed by micro-centrifugation, which was followed by quick freezing of the supernatants. The protein concentration was determined by using the Bio-Rad protein assay reagent according to the manufacturer's instructions. A fixed amount (50 ㎍) of cellular protein from the treated and untreated cell extracts was separated using SDS-polyacrylamide gel electrophoresis, and was electroblotted onto a nitrocellulose membrane. The immunoblot was incubated overnight with a blocking solution, followed by incubation with dilution of polyclonal antibodies against TNF-α, IL-4, COX-2 and STAT6. The blots were washed twice with Tween20/Tris-buffered saline (TTBS) and incubated with diluted solutions of horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody. The blots were washed thrice with TTBS and then developed under enhanced chemiluminescence.

8) Statistical analysis

All experiments were repeated at least three times. All data were expressed as mean ± SEM values. Differences between the means of the individual groups were assessed by one-way analysis of variance with Duncan's multiple range tests. Differences were considered significant at $P < 0.05$. The statistical software package SPSS version 14.0 (SPSS Institute, Chicago, IL, USA) was used for these analysis.

RESULTS AND DISCUSSION

Inflammatory bowel disease (IBD) is a common chronic gastro-intestinal disorder characterized by alternating periods of remission and active intestinal inflammation. The precise etiology of IBD, including Crohn's disease (CD) and ulcerative colitis (UC), remains unclear. However, environmental factors, immunological disturbances, genetic influences and the presence of certain chemical mediators (cytokines) have been established as putative participants in the pathogenesis of the disease (Lashner *et al.*,1995; Barbieri *et al.*, 2000; Podolsky *et al.*, 2002). In the last few decades, the development of experimental models for studying IBD has greatly contributed to enhance understanding of the immunological mechanisms involved, such as changes in the gut epithelial barrier (Shorter *et al.*, 1972; Colpaert *et al.*, 2001).

IBD seems to occur when luminal antigens from the bacterial flora stimulate the immune system in the gut barrier towards an exacerbated, genetically defined response. Patients present an increase in the amount of intestinal bacterial antigen compared to healthy individuals (Bonen and Cho, 2003). In particular, some human and animal studies have shown the prime importance of gut epithelial barrier integrity and changes that lead to deregulation of the immune system as a result of the loss of intestinal homeostasis (Somasundaram *et al.*, 1997).

Numerous studies have demonstrated that polysaccharides extracted from *Prunus mume* exhibit therapeutic properties, including immuno-stimulation, anti-infection, anti-tumor, wound-healing, and other therapeutic aspects.

Because weight gain and total food intake can be indicators of intestinal health, we monitored body weight and food intake for each of the 5 groups of mice, before and after treatment (Table 1). The average body weight of the DSS-induced group was less than the average body weight of the control group and the PM group has higher average body weight than the DSS-induced group. Also, the DSS-induced group had a lower food intake than the other groups. We also examined the weight of the spleen and liver in each of 5 groups of mice after final treatment. The spleen and liver weight were significantly increased in DSS-induced group and in PM group was lower than DSS-induced group, as shown in Table 2.

We investigated whether PM mixture could affect serum IgA and IgE levels as measured by ELISA is shown in (Fig. 1). As showed that PM mixture drastically decreased IgA and IgE concentrations in serum. (Wasser *et al.*, 2002) reported that

Table 1. Food intake and body weight of mice fed PM mixture for 15 days.

	Initial body weight (g)	Final weight (g)	Food intake (g)
A	21.80±0.84[a]	24.20±0.84[a]	17.40±1.52[a]*
B	22.20±0.84[a]	25.60±1.14[a]	19.20±1.64[a]
C	21.67±1.21[b]	20.00±0.98[c]	11.00±1.10[c]
D	21.40±0.55[b]	24.40±1.14[a]	15.50±2.06[b]
E	21.80±0.84[a]	23.40±1.14[b]	14.60±1.82[b]

*Data are mean±SEM of 5mice. Values denoted by different letters a-c are significantly different ($p < 0.05$).
A: Normal, B: PM administration, C: commercial diet + induced by DSS, D: induced by DSS + PM administration, E: induced by DSS + Sulfasalazine administration

Table 2. Effect of PM mixture on organs weight.

	Spleen (g)	Liver (g)
A	0.0768±0.0036[a]	0.9292±0.1069[a]*
B	0.0856±0.0035[a]	0.9340±0.0804[a]
C	0.1005±0.0141[a]	0.9932±0.0814[a]
D	0.0830±0.0048[a]	0.9484±0.1042[a]
E	0.0870±0.0050[a]	0.9660±0.0700[a]

*Data are mean±SEM of 5mice. Values denoted by different letters a-c are significantly different ($p < 0.05$).
A: Normal, B: PM administration, C: commercial diet + induced by DSS, D: induced by DSS + PM administration, E: induced by DSS + Sulfasalazine administration

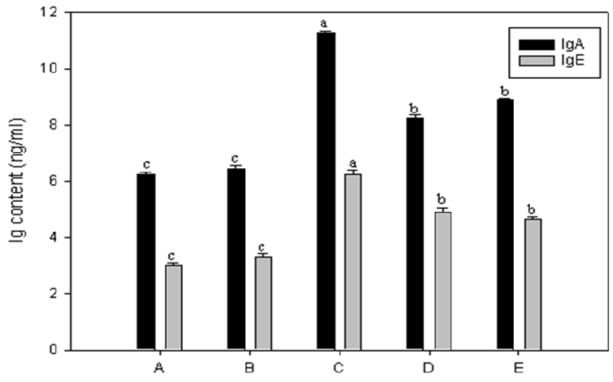

Fig. 1. The effect of PM on IgA and IgE production in serum. Antibody concentrations are in ng/㎖. Data are mean ±SEM of 5mice. Values denoted by different letters a-c are significantly different ($p < 0.05$).
A: Normal, B: PM administration, C: commercial diet + induced by DSS, D: induced by DSS + PM administration, E: induced by DSS + Sulfasalazine administration.

mushroom polysaccharides are known to stimulate natural killer cells, T-cells, B-cells, and macrophage-dependent immune system responses. They suggested that mushroom polysaccharides do not attack cancer cells directly, but produce their anti-tumor

Table 3. Effect of dietary PM mixture on spleen T-lymphocyte subsets.

	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$
A	17.10±1.69[a]	7.83±0.46[b]	2.18±0.17[b]*
B	16.07±1.07[a]	6.44±0.70[b]	2.52±0.33[a]
C	8.09±1.21[c]	10.50±1.3[a]	0.77±0.05[c]
D	13.87±2.21[b]	5.57±0.78[c]	2.51±0.36[a]
E	12.08±0.69[b]	4.82±0.64[c]	2.54±0.48[a]

*Data are mean±SEM of 5mice. Values denoted by different letters a-c are significantly different ($p < 0.05$).
A: Normal, B: PM administration, C: commercial diet + induced by DSS, D: induced by DSS + PM administration, E: induced by DSS+Sulfasalazine administration

Table 4. Effect of dietary PM mixture on MLN T-lymphocyte subsets.

	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$
A	45.71±3.37[a]	17.08±0.87[c]	2.68±0.25[a]*
B	46.92±2.66[a]	18.28±0.58[b]	2.57±0.22[a]
C	41.10±1.90[b]	21.69±1.93[a]	1.91±0.18[c]
D	45.13±2.00[b]	18.32±1.63[b]	2.47±0.13[a]
E	42.29±1.70[b]	18.22±1.26[b]	2.33±0.20[b]

*Data are mean±SEM of 5mice. Values denoted by different letters a-c are significantly different ($p < 0.05$).
A: Normal, B: PM administration, C: commercial diet + induced by DSS, D: induced by DSS + PM administration, E: induced by DSS + Sulfasalazine administration

effects by activating different immune responses in the host. Our results as showed that PM mixture drastically decreased IgA and IgE concentrations in the spleen, MLN, and PP lymphocytes.

There was a significant increase in the relative population of $CD4^+$ cells in the PM mixture group compared to the DSS groups. Also the ratio of $CD4^+/CD8^+$ T cells is significantly higher in the DSS group than the control and PM mixture groups in spleen lymphocytes. We observed a significant decrease in the relative population of $CD4^+$ T cells in the DSS group compared to the control and PM mixture groups and a significant decrease in the relative population of $CD8^+$ T cells in the PM mixture group compared to the DSS groups. Finally, the proportion of $CD4^+/CD8^+$ T cells was significantly higher in the DSS group compared to the control group and PM mixture group in the MLN lymphocytes (Tables 3 and 4).

It is well-known that certain cytokines, including TNF-α and COX-2 are important for intiating, regulating, and perpetuating inflammation in IBD. The expression of TNF-α in the spleen was higher in the DSS group than the PM mixture group. Also, the expression of COX-2 was significantly decreased in the treated group compared to the DSS group (Fig. 3). Next, we

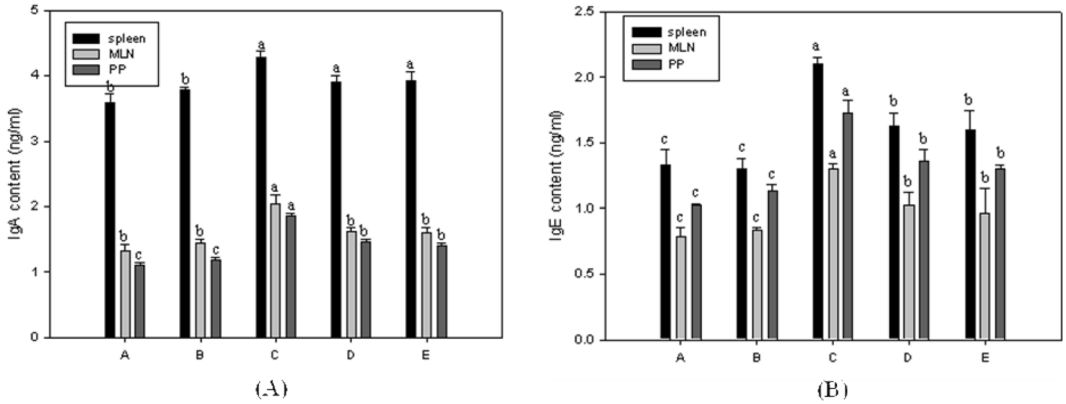

Fig. 2. The effect of PM on IgA (A) and IgE (B) production in the mouse spleen, MLN, PP. Antibody concentrations are in ng/㎖. Data are mean±SEM of 5mice. Values denoted by different letters a-c are significantly different ($p < 0.05$).
A: Normal, B: PM administration, C: commercial diet + induced by DSS, D: induced by DSS + PM administration, E: induced by DSS + Sulfasalazine administration.

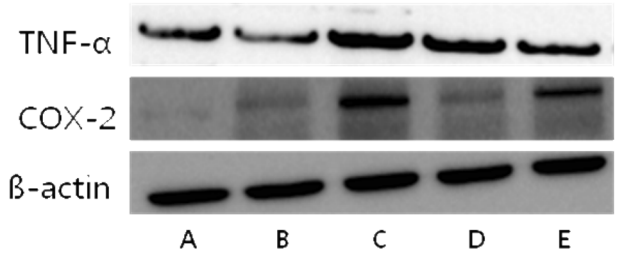

Fig. 3. The effect of PM on TNF-α and COX-2 expression in the mouse spleen. Expression of TNF-α and COX-2 protein was determined by Western blot analysis. Actin was used as an internal loading control.
A: Normal, B: PM administration, C: commercial diet + induced by DSS, D: induced by DSS + PM administration, E: induced by DSS + Sulfasalazine administration.

compared Th1 and Th2 cytokine expression to determine whether there was a bias for cellular (Th1) or humoral (Th2) immune response in IBD due to induced by DSS. Th1 and Th2 cytokines in DSS-induced mice were significantly decreased in PM mixture as shown in Fig 4. The expression of IFN-γ and STAT1 proteins in the spleen was significantly lower in the PM mixture group than the DSS group. Similarly, IL-4 and STAT6 decreased to significantly lower levels in the PM mixture group than the DSS group (Fig. 4).

Non-steroidal anti-inflammatory drugs (NSAIDs) produce or worsen the ulcerogenic response, in addition to impairing the healing of pre-existing lesions in the gastro-intestinal (GI) tract (Mizuno *et al.*, 1997; Ukawa *et al.*, 1998; Shigeta *et al.*, 1998; Halter *et al.*, 2001). These effects of NSAIDs are associated with a deficiency of endogenous prostaglandins (Pgs) due to the inhibition of cyclooxygenase (COX) activity. COX exists in two isoforms; COX-1 is observed constitutively expressed in various tissues, whereas COX-2 does not appear to be expressed except at very low levels in most tissues and is rapidly up regulated in response to growth factors and cytokines, such as tumor necrosis factor-α (TNF-α) (Feng *et al.*, 1993; Kargman *et al.*, 1996; O'Neill and Ford-Hutchinson 1993; Singer *et al.*, 1998). More recently, COX-2 has been implicated in several distinct cellular

Fig. 4. The effect of PM on IL-4, STAT6 (A) and IFN-γ, STAT1 (B) expression in the mouse spleen. (A) Expression of IL-4 and STAT6 protein was determined by Western blot analysis. (B) Expression of IFN-g and STAT1 protein was determined by Western blot analysis. Actin was used as an internal loading control.
A: Normal, B: PM administration, C: commercial diet + induced by DSS, D: induced by DSS + PM administration, E: induced by DSS + Sulfasalazine administration.

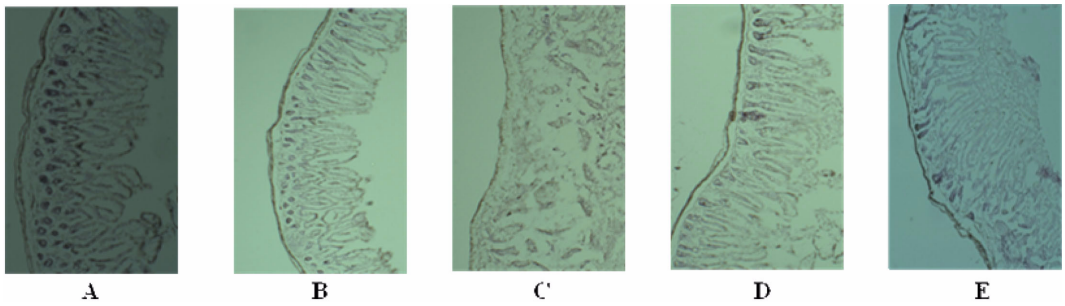

Fig. 5. Administration of PM attenuates the pathophysiological outcomes of colitis in DSS-induced mice. A assess histological damage of the crypt of colon, normal regular fed or induced by DSS mice with or of PM (50 ㎎/㎏) was administered to mice for 15 days. The section of colon sampled sacrificed mice on day-15 was stained with H&E.
A: Normal, B: PM administration, C: commercial diet + induced by DSS, D: induced by DSS + PM administration, E: induced by DSS + Sulfasalazine administration.

mechanisms, such as angiogenesis, proliferation and the prevention of apoptosis (Dempke *et al.*, 2001). New anti-inflammatory drugs have been synthesized, such as selective COX-2 inhibitors (anti-COX-2); however, these drugs may present side effects, such as the ability to modify the epithelial barrier. Recently, lumiracoxib, a novel highly selective COX-2 inhibitor was developed. The drug interacts with the COX-2 enzyme via mechanisms different from other COX-2 selective inhibitors. Lumiracoxib is associated with improved gastro-intestinal tolerability compared with non-selective COX inhibitors (Esser *et al.*, 2005; Rordorf *et al.*, 2003).

TNF-α is not only a major inflammatory cytokine and a powerful anti-cancer cytokine (Balkwill *et al.*, 2009), but TNF-α also induces a pro-inflammatory responses (Balkwill *et al.*, 2009), and TNF-α concentrations were found to be elevated in sera of children with active ulcerative colitis (UC) and colonic crohn's disease (CD) and in stools of children with both types of IBD (Murch *et al.*, 1991). Increased production of IL-1, TNF-α and IL-6 is observed even in microscopically normal CD mucosa, and cytokine profiles change during clinical evolution (Fiocchi *et al.*, 1998). However, Reinecker *et al*. (1993) reported that production of TNF-α is greater in cultures of CD than UC mucosal mononuclear cells. East and Isacke (2002) reported that IFN-γ is a pro-inflammatory cytokine that activates macrophages during inflammation, while IL-4 is associated with the resolution of inflammation. Liu *et al*. (2008) also reported that the production of IFN-γ dramatically increased, whereas IL-4 decreased in rats with colitis compared to the healthy rat. They suggested that mushroom polysaccharide decreased the production of IFN-γ and increased the production of IL-4 by macrophages and restored the condition in colitis similar to the control in vivo (Liu *et al*., 2008). However, Desreumaux *et al.* (1997) suggested that the elevation of IL-4 mRNA levels had distinct patterns in acute vs. chronic inflammation and Schreiber *et al*. (1995) also reported that the down-regulatory effect of IL-4 on activated circulating mononuclear cells is attenuated in IBD. The reason for this difference is not fully understood, but distinct immune regulatory mechanisms, genetically conditioned differences of mice models, or the degree of severity of the disease may be influences (Kim *et al.*, 2006).

The occurrence of IBD was confirmed on the basis of histological damage and inflammatory infiltrate as shown in (Fig. 5). The most severe IBD lesions were apparent in the DSS group and the PM mixture group showed fewer IBD lesions compared to the DSS group (Fig. 5).

The purpose of this study was to investigate the immuno-logical effect of dietary PM treatment in mice with inflammatory bowel disease (IBD) caused by DSS treatment. We demonstrate that PM mixture has anti-inflammatory effects in DSS-induced mice. Thus, we assume that PM mixture can prevent the development of inflammatory bowel disease (IBD) in mice. This is because PM mixture has additional anti-inflammatory effects mediated through reduced production of potent pro-inflammatory mucosal cytokines.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Education, Science and Technology and Korea Industrial Technology Foundation (KOTEF) through the Human Resource Training Project of Regional Innovation and also supported by Business for Cooperative R&D between Industry (Grants No. 00041100), Academy, and Research Institute funded Korea Small and Medium Business Administration in 2010.

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