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Evaluation of Anti-Colitic Effect of Chung-Jang-Hwan (C-mix) in Mice

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Abstract

The inhibitory effect of Chung-Jang-Hwan (C-mix) consisted of *Geranium nepalense* subsp. *thunbergii, Saururus chinensis,* and *Rubus coreanus* were investigated in dextran sulfate sodium (DSS)-induced colitic mice by microarray analysis. Treatment with C-mix improved colitic symptoms, including colon shortening and myeloperoxidase activity. Treatment with DSS alone upregulated the expression levels of inflammation-related genes, including *IL-1β, IL-6, CCL2, CCL4, CCL5, CCL7, CCL8, CCL24, CXCL1, CXCL2, CXCL5, CXCL9* and *CXCL10,* and other colitis-related genes such as *COX-2, PAP, MMP* family, *S100a8, S100a9* and *DEFA1* in mice. However, treatment with C-mix inhibited the expression levels of inflammation-associated genes induced by DSS. The increased expression levels of *COX-2* and *IL-1β*, representative inflammatory genes, were confirmed by a quantitative real-time polymerase chain reaction analysis. These results indicate that C-mix may ameliorate colitis by the inhibitory regulation of inflammation-associated genes.

Key Words: Chung-Jang-Hwan, C-mix, Colitis, Inflammation, Microarray

INTRODUCTION

Inflammatory bowel diseases (IBD) are generally separated into two types of disease--ulcerative colitis (UC) and Crohn's disease (CD)--on the basis of their clinical characteristics. IBD is a chronically relapsing disorder of the intestine accompanied by an increment of neutrophil influx and the production of inflammatory mediators (Shanahan, 2002; Binder, 2004). The pathogenesis of IBD is regarded as the result of a dysregulation of the intestinal immune response to a variety of antigens, such as intestinal microbes, and is characterized by T cell-rich infiltrates and an increment of *IL-6* in the inflamed mucosa of the colon, wherein many intestinal microbes reside (Rafii *et al.,* 1999; Atreya *et al.,* 2000). The pathogenesis of IBD may be associated with the upregulated expression of pro-inflammatory cytokines, such as *IL-1β* and *IL-6*, as well as other mediators that induce inflammatory activation of the mucosal immune system (Szkaradkiewicz *et al.,* 2009).

Inflammation-associated genes, such as pro-inflammatory cytokines, CXC and CC chemokines, and matrix metalloproteinases (*Mmp*), are upregulated in UC patients, according to the results of microarray analysis (Lawrance *et al.,* 2001; Salmela *et al.,* 2002; Yang *et al.,* 2002; Costello *et al.,* 2005). Chemokines regulate leukocyte trafficking during inflammation, as well as homeostatis (Zhong *et al.,* 2008). The migration of leukocytes into inflammatory sites is essential for the pathogenesis of colitis. *Cxcl1* belongs to the CXC chemokine family and was previously referred to as the GRO1 oncogene and/or GROα. It is expressed by macrophages, neutrophils and epithelial cells, and harbors a strong chemo-attractant for neutrophils (Jönsson *et al.,* 2009). Neutrophils are the first to be recruited to an infection and/or disease site. Among a variety of inflammatory mediators, CXC chemokines including *CXCL1* and *CXCL2* are the most critical for such recruitment (Kobayashi, 2008). *Cxcl5*, an epithelial-derived neutrophil-activating peptide 78, is produced following the stimulation of the inflammatory cytokine *IL-1β*. Increased *CXCL5* expression has been associated with neutrophil influx under inflammatory conditions (Amoli *et al.,* 2005). *CXCL9* has been identified as a monokine induced by gamma interferon (MIG) and has the

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ability to act as a T-cell chemo-attractant. The pro-inflammatory *CXCL1* and *CXCL9* are expressed concomitantly in UC patients (Egesten *et al.,* 2007). *CXCL10*, interferon-gamma-inducible protein-10 (IP-10), promotes the migration of activated T cells. Many studies have reported abnormal *CXCL10* expression in patients with UC and pouchitis (Uguccioni *et al.,* 1999; Autschbach *et al.,* 2002; Helwig *et al.,* 2004).

A large number of studies have focused on *MMPs* expression in the mucosa of patients with IBD as a marker for disease activity and potential therapeutic target (Baugh *et al.,* 1999; Louis *et al.,* 2000). *MMPs* are involved in tissue remodeling, angiogenesis, and the promotion of leukocyte extravasation in the actively inflamed areas of both UC and CD.

As a part of our studies on anticolitic agents from Korea folk medicines used for gastritis and diarrhea, we found that Chung-Jang-Hwan (C-mix), which is consisted of the dried aerial parts of *Geranium nepalense* subsp. *thunbergii* (family Geraniaceae) and *Saururus chinensis* (family Saururaceae) and the fruit of *Rubus coreanus* (family Rosaceae) potently inhibited dextran sulfate sodium (DSS)-induced colitis in mice. Therefore, in this study, we evaluated its anti-inflammatory and preventive effects in DSS-induced colitic mice by microarray and quantitative real-time polymerase chain reaction (qPCR) analysis.

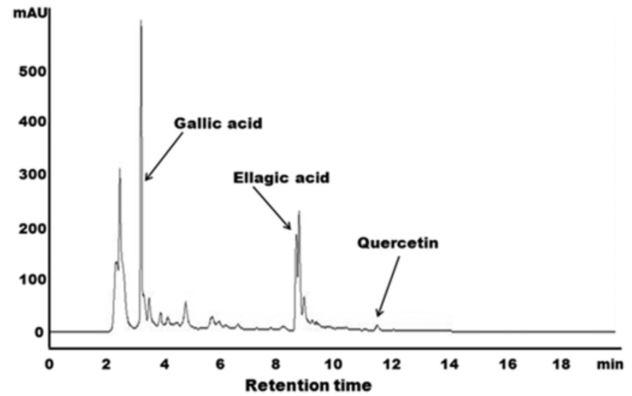

Fig. 1. HPLC chromatogram of C-mix. HPLC analyses were performed on the HPLC (Younglin high performance liquid chromatography system): column, Agilent zorbox C-18 (4.6 mm i.d.×150 mm, 5.8 μm particle diameter); mobile phase, the mixture of 0.5% phosphoric acid in water and 100% acetonitrile (85:15 for 0-5 min, 65:35 for 5-10 min, and 85:15 for 10-20 min); injection volumne, 10 μl; flow rate, 0.9 ml/min; temperature, 40°C; and detection, UV at 280 nm.

MATERIALS AND METHODS

Preparation of herbal mixtures

The dried aerial parts of *Geranium nepalense* subsp. *thunbergii* (family Geraniaceae) and *Saururus chinensis* (family Saururaceae) and the fruit of *Rubus coreanus* (family Rosaceae), which was purchased at KyungDong Herbal Market, Seoul, Korea, were identified by adjunct Professor Nam-Jae Kim, Kyung Hee University. The voucher specimens were deposited at the herbarium of College of Pharmacy, Kyung Hee University, Seoul, Korea. Each herbal medicine (0.5 kg) was extracted by boiling them in distilled water for 3 h respectively, filtered through a nylon mesh, and freeze-dried. Their yields were 2.9, 6.1 and 3.1%, respectively. Total phenolic contents of the aqueous extracts of *Geranium nepalense* subsp. *thunbergii, Saururus chinensis* and *Rubus coreanus* were 3.9, 5.7 and 7.7%, respectively. Total phenolic content was spectrophotometrically assessed at 760 nm using Folin-Ciocalteu reagent (Singleton *et al.*, 1999) and expressed as tannic acid equivalents. C-mix was prepared by mixing identical amounts (w/w) of the three extracts: its content of gallic acid, ellagic acid and quercetin are 113.3 ± 2.6, 45.3 ± 0.8 and 6.4 ± 0.1 μg/ml, respectively, by HPLC analysis (Fig. 1).

HPLC analyses were performed on the HPLC (Younglin high performance liquid chromatography system): column, Agilent zorbox C-18 (4.6 mm i.d.×150 mm, 5.8 μm particle diameter); mobile phase, the mixture of 0.5% phosphoric acid in water and 100% acetonitrile (85:15 for 0-5 min, 65:35 for 5-10 min, and 85:15 for 10-20 min); injection volumne, 10 μl; flow rate, 0.9 ml/min; temperature, 40°C; and detection, UV at 280 nm.

Animals

Male ICR mice (20-22 g, aged 4 weeks) were supplied by the Central Laboratory Animal Inc. (Seoul, Korea). All mice were maintained under controlled conditions of humidity (50 ± 10%), light (12/12 hr light/dark cycle), and temperature (25 ± 2°C), fed on standard laboratory chow (Samyang, Seoul, Korea) and permitted *ad libitum* access to water. All procedures related to animals and their care were approved by the Institutional Animal Use Committee.

Animal treatment protocol

Acute colitis was induced by treatment with 2% DSS (molecular weight 36,000-50,000 Da, MP Biochemicals, Solon, OH, USA) in drinking water for seven days. DSS-induced colitic mice were prepared according to the method described by Fukata *et al.* (2006). Each group consisted of seven mice. The mice were divided randomly into three groups - a normal group treated with drinking water containing the vehicle alone, a DSS group treated with DSS alone, and a DSS/C-mix group treated with DSS and C-mix (18 mg in 500 μl PBS). In the C-mix treatment group, the oral administration of C-mix started seven days prior to DSS treatment and continued for up to seven days with DSS treatment, so the C-mix treatment continued for a total of 14 days. In control group, vehicle alone was treated. The mice were anesthetized with ether and sacrificed on the seventh day after DSS treatment. The colons were quickly removed, opened longitudinally, and washed gently in PBS. The colon tissue was then employed for microarray analysis. The mouse colons were stored at −80°C until being used in the experiment.

Macroscopic and histological analysis

Three representative intestine tissue strips from regions 1, 3, and 7 cm proximal to the anus were obtained for macroscopic and histological analysis from each animal. The paraffin embedded tissue sections (3 or more per colon) were hematoxylin-eosin stained, examined, and scored on a scale of 0-5 according to the methods described by Tipoe *et al.* with slight modification (Tipoe *et al.,* 1992). Statistical analysis was conducted using ANOVA followed by *post hoc* multiple comparison tests and *p*-values of <0.05 were considered statisti-

cally significant.

Determination of myeloperoxidase activity

Plasma myeloperoxidase (MPO) activity was evaluated using a mouse MPO ELISA kit in accordance with the manufacturer's instructions (Hycult Biotechnology, The Netherlands). In brief, 96-well plates were coated with anti-mouse monoclonal MPO antibody. 100 μl of serial dilutions of the standard or colon homogenate samples were added to each well and incubated for 2 h at room temperature. After four washes, 100 μl of peroxidase-conjugated streptavidin solution was added to each well, and the plates were incubated for 1 h at room temperature. The plate was again washed four times, then maintained for 30 min at room temperature to allow reaction with 100 μl of TMB substrate solution. The reaction was halted via the addition of 100 μl of blocking solution, and the absorbance at 450 nm was read with a microplate reader (Synergy 2, Bio-Tek, USA). The results were expressed as arbitrary units of relative value.

Microarray analysis

RNA was extracted from the mouse colons (30 mg) using an RNeasy Mini Kit (Qiagen, USA). The quantity and quality of the isolated RNA were measured via UV spectrophotometry, real-time PCR (ABI 7500, USA), and agarose gel electrophoresis (data not shown).

Target preparation: 2 μg of total RNA were added to mixture in a final volume of 12 μl, containing T7 oligo dT primer. The mixture was incubated for 10 min at 70°C and then chilled into ice for 5 min. Two microliter of 10X first-strand buffer, 4 μl of 5 mM dNTP mix, 1 μl of RNase inhibitor (20 U/μl) and 1 μl of Superscript™ II RNase H⁻ reverse transcriptase (200 U/μl) were added into mixture to make a final volume of 20 μl. The reaction mixture was incubated for 2 h at 42°C. To synthesize second-strand cDNA, 10 μl of 10X second-strand buffer, 63 μl of nuclease free water, 4 μl of 5 mM dNTP mix, 2 μl of DNA polymerase mix (20 U/μl) and 1ul of RNase H (2 U/μl) were added into the mixture of 20 μl. The reaction mixture was incubated for 2 h at 16°C. The dsDNA was purified and then we adjusted the volume of dsDNA solution using drying the solution or adding the nuclease free water to make a final volume of 14 μl. Four microliter of 10X reaction buffer, 12 μl of ATP, CTP, GTP mix (25 mM), 3ul of UTP (50 mM), 3 μl of amino allyl UTP and 4 μl of T7 enzyme mix were added into 14 μl of dsDNA solution for the IVT (*in vitro* transcription). The reaction mix was incubated for 14 h at 37°C and then cRNA was purified. cRNA yield was quantified by measuring the UV absorbance at 260 nm, and 3 μg of cRNA for each sample was coupled with Cy-dye (Amersham Pharmacia, Uppsala, Sweden). Cy-dye labeled cRNA (target) was fragmented for hybridization. Fragmentation resulted typically in a fragmented target with a size range between 100 and 200 bases.

Array hybridization: Fragmented target was used for hybridization of Agilent oligo microarray. Fragmented target was added into 2X hybridization buffer from In situ Hybridization kit of Agilent. Hybridization solution was applied onto Agilent oligo microarray, and then microarray was hybridized for 16 h at 60°C. Microarray was washed with wash solution I (6X SSC, 0.00005% Triton-X 102) for 10 min at room temperature, and then microarray was washed with wash solution II (0.1X SSC, 0.00005% Triton-X 102) for 10 min at 4°C.

Data analysis: Microarray was scanned with a GenePix 4000B scanner (Axon Instruments, San Diego, CA, USA), and the scanned image were analyzed using GenePix v6.0 software to gene expression ratio. Raw data was normalized by locally weighted scatter-plot smoother (LOWESS) normalization method.

Table 1. Primer sequences used for qPCR anaysis

GENE	Gene bank #	Primers (5'-3')
β-actin	NM_007393	F: AGAGGGAAATCGTGCGTGAC R: CAATAGTGATGACCTGGCCGT
IL-1β	NM_008361	F: CTTCCCCAGGGCATGTGA R: ACCCTGAGCGACCTGTCTTG
COX-2	NM_011198	F: GGTGGAGAGGTGTATCCCCC R: ACTTCCTGCCCCACAGCA

Quantitative real-time PCR analysis

qPCR analysis was conducted using the SYBR Green PCR Master Mix and RT-PCR kits (Applied Biosystems, USA) in accordance with the manufacturer's recommended protocols. In brief, PCR reactions were conducted in a reaction mixture (20 μl) containing 20 ng of RNA, 10 μl of SYBR Green PCR Master Mix, 6.25 U of MultiScribe reverse transcriptase, 10 U RNase inhibitor, and 0.1 mmol/L primers. The sequences of the primers used and the accession numbers of the genes analyzed are summarized in Table 1. The amplification conditions were 30 min at 48°C, 10 min at 95°C, and 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. For relative quantitation, we compared the amount of target normalized to an endogenous reference, β-actin. The formula $2^{-\Delta\Delta Ct}$ represents the n-fold differential expression of a specific gene in a treated sample as compared with the control, where Ct is the mean of the threshold cycle, ΔCt is the difference in Ct values for the target gene and the reference gene, β-actin (in each sample), and ΔΔCt represents the difference between the Ct from the control and each datum. We validated this method by first comparing the standard curves of the reference and the target to demonstrate that the efficiencies were equal.

Statistical analysis

All data are expressed as the means ± standard deviation (SD) with the statistical significance analyzed using Mann-Whitney U-test as well as student t-test. When we compared significance between two groups (normal vs. DSS group and DSS vs. DSS/C-mix group), the histological scores were all significant ($p<0.05$).

RESULTS

To evaluate the anticolitic effect of C-mix in mice, we treated it in DSS-induced colitic mice and measured body weight, colon length and myeloperoxidase activity (Fig. 2). During the experimental period, food intake was similar in animals in the normal, DSS, and DSS/C-mix groups (data not shown). The body weight loss was not significant on the seventh day after DSS treatment (Fig. 2A). The total colonic length was signif-

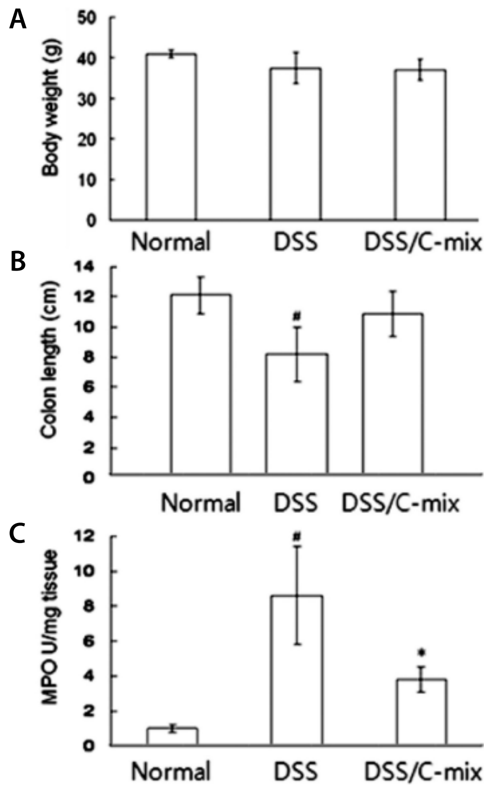

Fig. 2. C-mix treatment reduces the colitic symptoms. (A) The body weight was similar in the Normal, Dextran sulfate sodium (DSS), and C-mix groups on the seventh day after DSS treatment. (B) The colonic length was significantly shorter in the DSS group. (C) Colon MPO activity from the DSS group was higher than was detected in the normal and C-mix group. Each value indicates the mean ± S.D. (n=7). #Significantly different vs. normal group (*p*<0.05), *Significantly different vs. DSS (control) group (*p*<0.05).

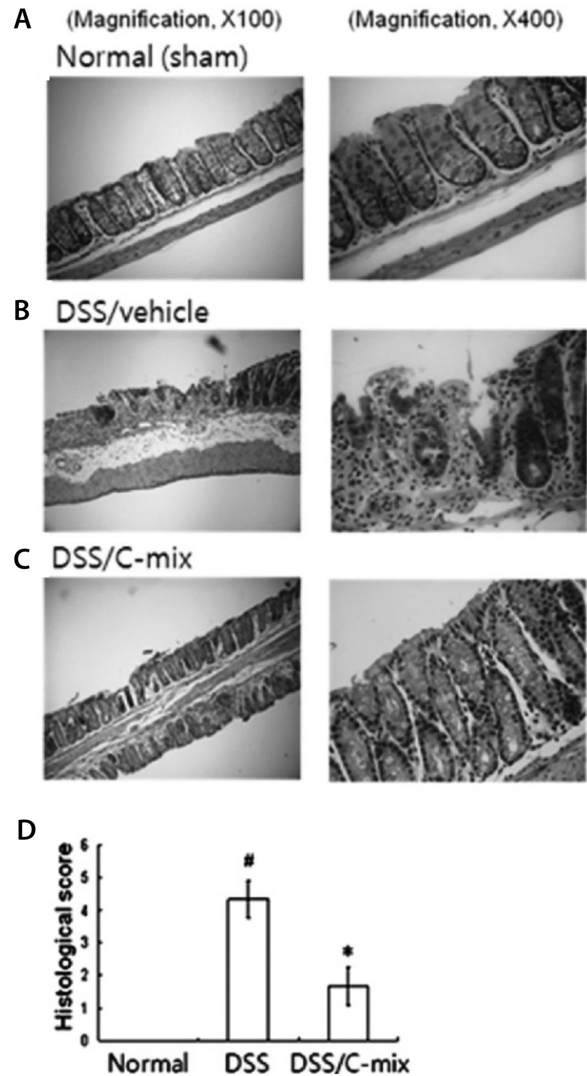

Fig. 3. C-mix treatment reduces the severity of Dextran sulfate sodium (DSS)-induced colitis in mice. (A) Distal colon sections stained with hematoxylin-eosin showed normal crypt morphology in normal group mice. (B) DSS treatment for 7 days induced inflammatory infilterates and crypt damage. (C) Oral administration of C-mix with DSS ameliorated crypt morphology. (D) Histological score suggested that the C-mix treatment reduced the severity of DSS-induced colonic damages. Each value indicates the mean ± S.D. (n=5). #Significantly different vs. normal group (*p*<0.05), *Significantly different vs. DSS (control) group (*p*<0.05).

icantly shorter in the DSS group than in the normal group. However, treatment with C-mix inhibited the colon shortening induced by DSS (Fig. 2B). Treatment with DSS alone also induced higher activity of MPO, an inflammatory marker, in colon epithelial cells. Colon MPO activity from the DSS group was higher than was detected in the normal group. By way of contrast, colon MPO activity in the DSS with C-mix group was reduced as compared with the DSS group (Fig. 2C).

When tissue sections of the colon were examined, seven days after the induction of colitis by DSS-treatment, completely destroyed mucosa without regular villi and destroyed intestinal micro-architecture were noted. Additionally, we noted widespread leukocyte infiltration, infiltrations of polymorphonuclear cells into the lamina propria, swallow erosion, and deep erosion, which were representative of extensive mucosal and/or transmural ulceration in the DSS-induced colitis group. However, the C-mix treatment group 1 week prior to DSS challenge (total 2 weeks administration, 1 week+1 week with DSS challenging) significantly reduced the DSS-induced colitic damage (Fig. 3). The microarchitecture was restored upon the administration of C-mix treatment. Although the presence of focal mucosal ulceration, losses of surface epithelium, infiltrations of a few polymorphonuclear cells and leukocytes from C-mix treated animals were noted, the extent of tissue infiltration was markedly reduced.

Inflammation-associated gene expression levels were evaluated via microarray analysis of the colon samples from mice treated with DSS with or without C-mix. All genes associated with inflammation and intestinal function that were upregulated or downregulated (≥2-fold DSS vs. normal, DSS with C-mix vs. normal) were selected (Table 2). DSS treatment increased the expression level of cytokines, *IL-1β* and *IL-6*, and chemokines, *CCL2, CCL4, CCL5, CCL7, CCL8, CCL24, CXCL1, CXCLl2, CXCL5, CXCL9 and CXCL10,* and their receptors, *CCR3,* but reduced CCR7 expression. The co-administration of DSS with C-mix induced a reduc-

Table 2. Microarray analysis of colonic gene expression in mice treated with dextran sulfate sodium (DSS) alone or with DSS and Chung-Jang-Hwan (CJH)

Gene	DSS vs. Normal	DSS/CJH vs. Normal
IL-1β	9	3.5
IL-6	22.8	3.2
IL-18	0.9	0.8
CCL2	4.5	2.7
CCL4	6.7	2.7
CCL5	3.6	2.1
CCL7	3.7	2
CCL8	4.7	4.5
CCL21	1.3	1.2
CCL24	3.3	1.5
CXCL1	5	1.1
CXCL2	5.4	1.8
CXCL5	21.4	6.4
CXCL9	8.7	5.6
CXCL10	6.1	3.1
CCR3	3.4	2
CCR7	0.3	1
PAP	7.9	3.1
S100a8	15	2.6
S100a9	15.8	5.4
MMP3	23.5	7.5
MMP9	2.7	2
MMP10	5.5	4.2
MMP13	9.9	2.5
SERPINE1	8	2.8
DEFA1	6.3	2.1
COX-2	2.5	0.8

Values represent average fold change (3 array experiments) between DSS vs. Normal (first column) and DSS/CJH vs. Normal (second column).

tion in the expression of these cytokines, chemokines, and inflammation-associated genes, but reversed CCR7 expression (Table 2). However, treatment with C-mix did not affect the expression of CCL21, which is a receptor of CCR7, and *IL-18*. *MMP3, MMP9, MMP10,* and *MMP13* were upregulated in DSS-induced colitis, particularly *MMP3* and *MMP13*. However, C-mix treatment significantly inhibited *MMP3* and *MMP13*. Additionally, the administration of C-mix reduced the expression of Defensin alpha 1 (*DEFA1*), S100 calcium binding protein A8 (*S100a8*) and S100 calcium binding protein A9 (*S100a9*), all of which were increased by DSS treatment.

Both *IL-1β* and *COX-2* are representative inflammation-associated genes. They were identified and selected from the microarray analysis data, and their expression levels were confirmed via qPCR analysis (Fig. 4). The expression levels of both genes differed significantly between the normal and DSS-treated groups. However, C-mix treatment inhibited the upregulation of *IL-1β* and *COX-2* expression in DSS-induced colitic mice.

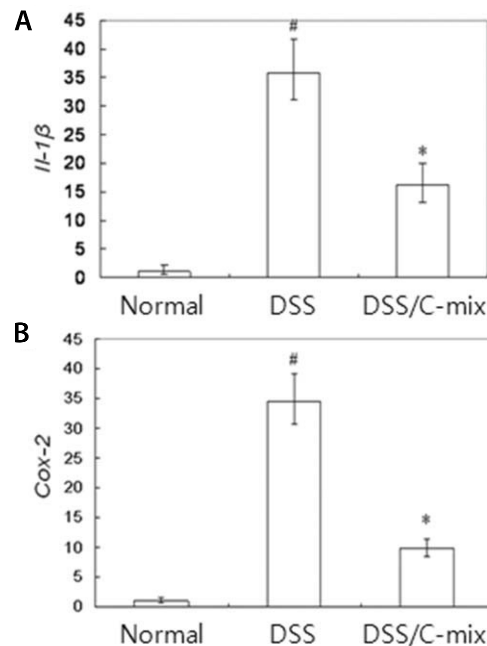

Fig. 4. C-mix inhibit DSS-induced IL-1β (A) and COX-2 (B) expression increased by dextran sulfate sodium (DSS) according to qPCR analysis. Each value indicates the mean ± S.D. (n=3). # Significantly different vs. normal group (p<0.05), *Significantly different vs. DSS (control) group (p<0.05).

DISCUSSION

Colon tissue sections from DSS-treatment mice revealed a completely destroyed mucosa without regular villi and destroyed intestinal micro-architecture. Oral administration of C-mix significantly improved DSS-induced colitis in mice: it inhibited colon shortening and myeoloperoxidase activity. These results suggest that C-mix may induce and/or inhibit inflammatory mediators. Therefore, we investigated the ability of C-mix to suppress inflammation-associated gene expression by microarray analysis, a powerful tool for determining the expression profile of a number of genes. Several microarray studies have reported that the observed gene expression could provide useful information regarding IBD and colon carcinogenesis (Lobenhofer *et al.,* 2001; Costello *et al.,* 2005). We also found that treatment with DSS alone increased the expression levels of pro-inflammatory cytokines, including *IL-1β* and *IL-6*, inflammation-associated CC chemokines, such as *CCL2, CCL4, CCL5, CCL7, CCL8,* and *CCL24,* CXC chemokines, such as *CXCL1, CXCL2, CXCL5, CXCL9,* and *CXCL10,* and their receptors, such as *CCR3,* as well as inflammation-associated enzymes, such as *COX-2, MMP3,* and *MMP3.* IL-1β and IL-6 are we known proinflammatory cytokines and CXCL 1 and 2 are essential for neutrophil recruitment. *CXCLs* are induced by inflammatory cytokines in human colonic enterocyte cells and pharyngeal epithelium and increased in the colons of IBD patients (Cole *et al.,* 2001; Kwon *et al.,* 2005; Egesten *et al.,* 2009). Additionally, the inhibition of *CXCL10* protects DSS-induced and *IL-10* knockout colitic mice (Shimoyama *et al.,* 2001; Sasaki *et al.,* 2002). *CCL4* is

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a major factor generated by macrophages after stimulation with bacterial endotoxins. It also induces the synthesis and release of other pro-inflammatory cytokines, such as *IL-1β* and *IL-6*. *CCL24* interacts with the *CCR3* chemokine receptor to induce chemotaxis in eosinophils (Pallone and Monteleone, 1998; Schreiber *et al.,* 1999; McCormack *et al.,* 2001; Kitamura *et al.,* 2004; Papadakis, 2004). Treatment with C-mix reduced these proinflammatory cytokines, chemokines, chemokine receptors expression induced by DSS in mice. These results suggest that C-mix may inhibit the expression of these inflammatory cytokines and chemokines by the regulation of CCL4 expression.

Overexpression of pancreatitis-associated protein (*Pap*) was detected in the colonic mucosa of IBD patients and DSS-induced colitic mice (Ogawa *et al.,* 2003). Additionally, gene members of the S-100 calcium binding protein family, endogenous activators of innate immune responses, overexpressed in IBD patients were increased, like the previously reports (Lawrance *et al.,* 2001; Dieckgraefe *et al.,* 2002; te Velde *et al.,* 2007). Treatment with C-mix reduced *Pap, S100a8* and *S100a9* expression. The majority of the *MMPs* are overexpressed in the same manner as previously reported in two experimental colitic mice models (Lawrance *et al.,* 2001) in particular *Mmp3*. Treatment with C-mix significantly inhibited *MMP3* and *MMP13* expression. These results suggest that C-mix may ameliorate colitic inflammation via the regulation of *MMPs* expression.

In this study, we found that treatment with C-mix significantly inhibited the expression of inflammation-associated genes, particularly *COX-2,* which is known as an inflammatory mediator in many diseases, and the pro-inflammatory cytokine *IL-1β* (Schreiber *et al.,* 1999; Khan *et al.*, 2002), by a microarray analysis. To confirm it, we measured the gene expression levels of *COX-2* and *IL-1β* via qPCR analysis. C-mix significantly reduced these genes expression. This result is supported by the previous reports that *Saururus chinensis* exhibits anti-inflammatory activity (Lee *et al.,* 2003; Yoo *et al.,* 2008), *Geranium nepalense* subsp. *Thunbergii* exhibits marked radical scavenging activity (Xiufen *et al.,* 2004), and *Rubus coreanus* suppresses the production of inflammatory mediators such as nitric oxide (NO), prostaglandin E2 (PGE2) and inflammatory cytokines (Park et al., 2006; Yang *et al.,* 2008).

Based on these findings, C-mix may ameliorate colitis by the inhibitory regulation of of inflammation-associated genes.

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