

# Effect of *Artemisiae Capillaris* Herba on Anti-inflammatory Properties in RAW264.7 Cell Line

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The present study was performed to examine the possible anti-inflammatory effects of a herbal drug ASCH in RAW264.7 cell line. Inflammation was induced by LPS toxin treatment to RAW264.7 macrophage cell line. Increases in cytokine production such as IL-1 $\beta$ , IL-6, and IL-18, COX-2, NOS-II (iNOS), and TNF-alpha were observed at mRNA level in the LPS-treated RAW264.7 cells. Measurement of IL-6, nitric oxide and the reactive oxygen species (ROS) showed increased production of these inflammation mediators. Treatment of ASCH effectively decreased IL-1 $\beta$  protein in a dose-dependent manner, and IL-6 and IL-18 were reduced at 100  $\mu$ g/ml of ASCH concentration. NO production was also decreased by ASCH treatment. A slight inhibition for TNF-alpha in terms of protein, but not mRNA level was obtained by 100  $\mu$ g/ml of ASCH treatment. ASCH treatment to normal RAW264.7 cells did not produce any cytotoxicity, indicating that the action of ASCH was selective to inflammatory cells. Thus, the present data suggest that ASCH may act as an important regulator to alleviate the inflammatory symptoms.

Key words : *Artemisiae Capillaris* Herba, RT-PCR, ELISA reader, RAW264.7, inflammation, cytokine, nitric oxide

## Introduction

The exogenous and endogenous noxious stimulations cause a complex reaction in vascularized connective tissues called inflammation, which is characterized by increased blood flow (vasodilation) and cellular recruitment and activation. Vascular changes involve increases in vascular diameter and flow, and increases in vascular permeability.<sup>25,38)</sup> This change is closely associated with the sequence of events in leukocyte emigration to the original stimulation area. The extravasation of leukocytes begins with margination, rolling and adhesion of the leukocytes in the capillaries.

Then the cells transmigrate into the interstitial fluid and then to the injury site by chemotaxis. Numerous chemical mediators are known to be involved during inflammation. Vasoactive amines such as histamine and serotonin are increased and cause arteriolar dilation and also increase vascular permeability. Neuropeptide such as substance P that

transmits pain signals, regulates vessel tone, and modulates vascular permeability. Arachidonic acid metabolites including prostaglandin, leukotrienes, and lipoxins can virtually mediate every step of inflammation. Because of the importance of arachidonic acid metabolism, numerous studies have been focused on the induction mechanism of cyclooxygenase (COX) that synthesizes prostaglandin.<sup>14,38,53)</sup>

There are two forms of COX, called COX-1 and COX-2, which are both important for mediating inflammation, and COX inhibitors such as aspirin and most nonsteroidal anti-inflammatory drugs (NSAID) such as ibuprofen have been used as an anti-inflammatory drug.<sup>7,26,34)</sup> Thromboxane A<sub>2</sub>, leukotrienes C<sub>4</sub>, D<sub>4</sub>, and E<sub>4</sub> induce vasoconstriction, and prostacyclin, prostaglandin, and lipoxins induce vasoconstriction, and leukotrien are also involved in increased vascular permeability, chemotaxis, and leukocyte adhesion.<sup>2,4,20,27)</sup> Plasma proteases (; kinin, clotting, fibrinolytic and complement cascades) and platelet activating factor (PAF) are also important for complement reactions, and for controlling inflammation by increasing leukocyte adhesion, and chemotaxis.

Besides diverse molecular factors described above, cytokines polypeptide products of many cell types are critical

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· Received : 2004/09/10 · Revised : 2004/10/20 · Accepted : 2004/11/17

for the regulation of inflammation. Cytokine secretion is transient and tightly regulated during inflammation. Interleukin 1 (IL-1) and tumor necrosis factor (TNF) are produced by activated macrophage<sup>2,12,37</sup> though IL-1 can also be synthesized by other cell types, and secretion is stimulated by endotoxin, immune complexes, toxins, physical injuries or a variety of inflammatory mediators.<sup>3,9,43</sup> Both IL-1 and TNF induce endothelial activation.<sup>39,53</sup> TNF also causes aggregation and activation of neutrophils and the release of proteolytic enzymes from mesenchymal cells, thus contributing tissue damage.<sup>12,18,23</sup> Besides IL-1 and TNF, other cytokines such as IL-6, 16 and others are known to mediate inflammation reactions.<sup>5,11,18,19,24,36</sup>

Nitric oxide synthase (NOS) is an enzyme catalyzing the production of nitric oxide (NO) which is involved in vasodilation and other diverse physiological effects. Of three types of NOS, iNOS (inducible NOS, also called NOS II) is present in endothelium, smooth muscle cells, macrophages, and cells in cardiac, respiratory and kidney cells. iNOS is induced by a number of inflammatory cytokines and mediators including IL-1, TNF, interferon-gamma and by lipopolysaccharide (LPS). NO plays multiple roles in inflammation including (i) relaxation of vascular smooth muscle (vasodilation), (ii) antagonism of all stages of platelet activation (adhesion, aggregation, degranulation), (iii) reduction of leukocyte recruitment at the inflammation sites, and (iv) action at a microbicidal agent.<sup>22,40,44</sup>

Synthesis of reactive oxygen species (ROS) such as superoxide and toxic NO derivatives is induced from neutrophils and macrophages after stimulation by chemotactic agent, immune complexes, or macrophage activation during inflammation.<sup>1,32</sup> At low levels, ROS can induce cytokine and chemokine and adhesion molecule expression, but at high concentration, they are involved in a variety of tissue injury mechanism.<sup>35,46,55</sup> Thus, diverse antioxidant mechanisms (e.g., catalase, superoxide dismutase and glutathione) are present in tissues and serum to minimize the toxicity of the oxygen metabolism.

In this study, potential anti-inflammatory effects of a herb plant *Artemisiae capillaris herba* (ASCH) were investigated in inflammation-induced macrophage cells. ASCH is obtained by drying and extracting *Artemisia capillaris* Thumb and *Artemisia scoparia* Waldst. This herb contains beta-pinene, capillin, capillone, capillarin, and also several fatty acids such as steric acid, palmitic acid, and oleic acid. Although the ASCH has been used in oriental medicine to control inflammation-related diverse diseases,<sup>28,29</sup> its specific effect on inflammation has not been studied. Our data in the in vitro experiments using RAW264.7 cells indicate that ASCH decreased chemical mediators which are critical for inflammation.

## Experimental procedures

### 1. Materials

#### 1) Experimental animals

C57BL/6 mice (6 week old, female) were adjusted in the animal room for 1 week and then used for the experiment. The animal room was maintained at 22±2°C with a 12 hr day and night cycle. Day time light intensity was 200-300 Lux. The composition of food pellets (Samyang Co., no antibiotics added) contains crude protein (higher than 22.1%), crude fat (less than 8.0%), crude fibers (less than 5.0%), crude minerals (less than 8.0%), calcium (less than 0.6%), phosphorus (higher than 0.4%). The food and water were supplied with no limitation.

#### 2) Reagents and apparatus

##### (1) Reagents

The reagents used in the present study are as follows; diethyl py- rocarbonate (DEPC), 3, 4, 5-dimethyl-thiazol-2,5-carboxymethoxyphenyl-2, 4- sulfophenyl-2H- tetrazolium (MTS), 2, 7,-dichlorodihydro-fluorescein diacetate (DCFH-DA), complete adjuvant, chloroform, RPMI-1640 medium, isopropanol, RBC lysis solution, ethidium bromide, dulbecco's phosphate buffered saline (D-PBS), formaldehyde, foramide, and magnesium chloride (MgCl<sub>2</sub>) were all purchased from Sigma (USA). Fetal bovine serum (FBS) was from Hyclone (Logan, Co., U.S.A), agarose from FMC (U.S.A), propidium iodide (PI) and RNase from Pharmingen (Torreyana., Co., U.S.A), and IL-6 and TNF-α, and ELISA kit from R&D system (Minneapolis., Co., U.S.A). Other chemicals were used with the highest quality available

##### (2) Apparatus

The instruments used in this study are as follows ; heat extractor (Daewoong, Korea), rotary vacuum evaporator (Büchi B-480, Switzerland), freeze dryer (EYELA FDU-540, Japan), CO<sub>2</sub> incubator (Forma scientific Co., U.S.A), clean bench (Vision scientific Co., Korea), autoclave (Sanyo, Japan), micropipet (Gilson, France), water bath (Vision scientific Co., Korea), vortex mixer (Vision scientific Co., Korea), spec- trophotometer (Shimadzu, Co., Japan), centrifuge (Sigma, Co., U.S.A), deep-freezer (Sanyo, Co., Japan), thermocycler system (MWG Biotech., Co., Germany), ice-maker (Vision scientific Co., Korea), homogenizer (OMNI, U.S.A), plate shaker (Lab-Line, Co., U.S.A) and ELISA reader (Molecular Devices, Co., U.S.A).

#### 2) Drug

The drug *Artemisiae Capillaris Herba* (ASCH) used in this study was obtained from Daejeon University Oriental medicine hospital and used after purification.

#### 3) Isolation of ASCH

150 g of ASCH were suspended in 2,000 ml of distilled water, heat extracted for 3 hr, and filtered. The filtrate was

concentrated by vacuum evaporation using the rotary evaporator (BUCHI B-480, Switzerland) and freezing drying with the freeze dryer (EYELA FDU-540, Japan). The product obtained was kept at -84°C, and used after appropriate dilution.

## 2. Methods

### 1) Isolation and culture of mouse lung fibroblast cells

Mouse lung tissues were dissected, washed with cold PBS three times and minced into small pieces. The tissues in 15 ml conical tube were centrifuged at 1400 rpm for 5 min. After adding DMEM containing collagenase A (5 mg/ml, BM, USA), DNase I (0.15 mg/ml, Sigma, USA), and antibiotics (penicillin 104 U/ml, streptomycin 10 mg/ml, amphotericin B 25 µg/ml), the dissociated tissue was incubated at 37°C for 2 hr, and further incubated for 30 min in the presence of 0.5% trypsin-0.2% EDTA. Then the tissue was washed with cold PBS twice, centrifuged at 1500 rpm to remove the supernatant, resuspended in DMEM-10% FBS, and cultured for 7 days. mLFC was separated by 0.5% trypsin-0.2% EDTA treatment, and cell concentration was adjusted to 10<sup>5</sup> cells/ml in DMEM-5% FBS and plated on 96 well plate. RAW264.7 cell line was plated with a concentration of 10<sup>5</sup> cells/ml in DMEM-10% FBS.

### 2) Measurement of cytotoxicity

The cytotoxicity was measured by modified MCB method. After incubation in 5% CO<sub>2</sub> incubator at 37°C for 1 hr, mLFC or mouse macrophage cells were treated with 200 µg/ml, 100 µg/ml, 50 µg/ml, 10 µg/ml, 1 µg/ml of ASCH for 48 hr. The culture medium was then removed and cells were washed with PBS twice. Cells in each well were treated with 50 µl of 50% TCA (trichloroacetic acid) for 1 hr at 4°C, washed with distilled water five times, and air dried. Cells were stained with MCB solution (0.4%/1% acetic acid ; 100 µl/well) for 30 min at room temperature, and washed with 0.1% acetic acid for 4-5 times. Cells were air dried, and solubilized in 10 mM Tris base (100 µl/well). Cells in the plate were dispersed in the culture medium on a plate shaker (Lab-Line, USA) for 5 min and used for the measurement of optical density at 540 nm using ELISA reader (molecular devices, USA).

### 3) RT-PCR of RAW264.7 cell line

#### (1) Total RNA extraction

RAW264.7 cells were plated on 24 well plate with a cell concentration of 1 × 10<sup>6</sup> cells/well. The cells were treated with 100 µg/ml, 10 µg/ml, 1 µg/ml of ASCH for 1 hr followed by LPS treatment (2 µg/ml) for 6 hr. Cells were then collected and mixed with 500 µl of RNAzolB for cell solubilization and 50 µl of chloroform (CHCl<sub>3</sub>) was added to the supernatant for 15 sec. Then the sample was incubated on ice for 15 min and centrifuged at 13,000 rpm to collect the supernatant. 200 µl of supernatant was mixed with an equal volume of 2-propanol

and incubated on ice for 15 min. The phase was separated again by centrifugation at 13,000 rpm and the pellet was washed with 80% of ethanol and vacuum dried for 3 min. Extracted total RNA was resuspended in 20 µl of diethyl pyrocarbonate (DEPC) water and used for reverse transcription-polymerase chain reaction (RT-PCR).

#### (2) RT-PCR

Total RNA (3 µg) was denatured for 10 min at 75°C. The denatured RNA was mixed with 2.5 µl 10 mM dNTPs mix, 1 µl random sequence hexanucleotides (25 pmole/25 µl), 1 µl RNasin (20 U/µl), 1 µl 100 mM DTT, 4.5 µl 5× RT buffer (250 mM Tris-Cl, pH 8.3, 375 mM KCl, 15 mM MgCl<sub>2</sub>), 1 µl M-MLV RT (200 U/µl), and H<sub>2</sub>O to bring up the reaction to 20 µl. The sample was mixed well and incubated for 60 min for the synthesis of first stranded cDNA, and the reaction was stopped by placing for 5 min at 95°C.

#### (3) cDNA PCR

Synthesized cDNA was used for PCR by using Primus 96 Legal PCR system with high pressure lid (MWG in Germany). Three microliters of cDNA template, 1.0 µl of sense and antisense primers (20 pmole/µl each) for β-actin, IL-1β, IL-6, IL-18, tumor necrosis factor-α (TNF-α), cyclooxygenase-2, and NOS-II, 3 µl of 2.5 mM dNTPs, 3 µl of 10× PCR buffer (100 mM Tris-Cl, pH 8.3, 500 mM KCl, 15 mM MgCl<sub>2</sub>), and 0.18 µl of Taq. polymerase (5 U/µl) were added to the reaction and the reaction volume was adjusted to 30 µl. The condition for PCR was pre-denaturation at 95°C for 5 min, annealing at 55°C for 1 min, elongation at 72°C for 1 min, and denaturation at 95°C for 1 min. After 30 cycles of the reaction, the sample was post-elongated at 72°C for 3 min. 20 µl of individual PCR products were analyzed by running gel for 20 min at 120 volts on a 1.2% agarose gel electrophoresis. PCR products on an agarose gel electrophoresis were quantitated by Windows 1D main program (AAB, USA) presenting the data as maximum height (Ht). The oligonucleotides used as primers were as follows;

Gene	Primer	Sequence
IL-1β	sense	5'-GCAACTGTTCCCTGAACTCA-3'
	antisense	5'-CTCGGAGCCTGTAGTGCAG-3'
IL-6	sense	5'-CCGTCGATAGTGGCATCCATGAAAC-3'
	antisense	5'-GGACCAATACCTGCTATAGGG-3'
TNF-α	sense	5'-AACCCCTACTGAACTCAGATTGTTAG-3'
	antisense	5'-TAAGTCAGTTTAAATGCTTAGGG-3'
IL-18	sense	5'-ACTgTACAACCGcAgTAATACgg-3'
	antisense	5'-AgTgAACATTACAgATTATCCC-3'
NOS-II	sense	5'-CCGATATTTAGATACGTTAAAC-3'
	antisense	5'-ATGGCCTAGTCAGTCTCTAAAT-3'
COX-2	sense	5'-TTCAAATgAgATTgTgggAAAAT-3'
	antisense	5'-AgATCATCTCTgCCTgAgTATCTT-3'
β-actin	sense	5'-TGGAAATCCTGTGGTCCATGAAAC-3'
	antisense	5'-GTCACAGTCAGCTGTATAGGG-3'

4) Measurement of cytokines

RAW264.7 cells ( $1 \times 10^5$  cells/ml) were subcultured for 3 days, and  $2 \times 10^5$  cells/well were plated on 96 well dishes and then treated with FBS-deleted RPMI 1640 medium. Cells were treated with ASCH (100  $\mu\text{g/ml}$ , 10  $\mu\text{g/ml}$ , 1  $\mu\text{g/ml}$ ) for 1 hr, and then with LPS (2  $\mu\text{g/ml}$ ) for 6 hr. Cells were washed with DMEM medium and added with fresh medium and cultured for 24 hr at 37°C CO<sub>2</sub> incubator. Culture supernatant was collected by centrifugation at 2000 rpm for 5 min and IL-6 and TNF- $\alpha$  proteins were determined by using enzyme-linked immuno-sorbent assay kits appropriate to individual proteins (ELISA, Endogen, USA).

5) Measurement of nitric oxide (NO) production

RAW264.7 cells were plated with  $2 \times 10^4$  cells per each well of 96 well plates. After treatment with 100  $\mu\text{g/ml}$ , 10  $\mu\text{g/ml}$ , 1  $\mu\text{g/ml}$  of ASCH for 1 hr, cells were cultured for 48 hr in the presence of LPS (2  $\mu\text{g/ml}$  per well). Then, the supernatant was taken by centrifugation at 2000 rpm for 5 min and used for NO measurement. The Griess reagents used for NO measurement were prepared as solution A (0.2% Naphthylethylene diamine dihydrochloride in water) and solution B (2% Sulfonamide in 5% H<sub>3</sub>PO<sub>4</sub>), kept in a cold, dark place, and mixed with 1:1 ratio before use. 100  $\mu\text{l}$  of culture supernatant was dispensed in 96 well plate and after adding 100  $\mu\text{l}$  of Griess mixture solution, the absorbance at 540 nm was measured using ELISA reader.

6) Measurement of intracellular reactive oxygen species (ROS)

Cells in 24 well plate with  $5 \times 10^5$  cells/well were treated with 100  $\mu\text{g/ml}$ , 10  $\mu\text{g/ml}$ , 1  $\mu\text{g/ml}$  of ASCH for 1 hr and cultured for 48 hr at 37°C in the presence of LPS (2  $\mu\text{g/ml}$ ). After culture, cells were treated with 50  $\mu\text{M}$  of DCFH-DA for 5 min, washed twice, and intracellular DCF or ROS was measured using flow cytometer (Becton Dickinson, USA).

Results

1. Determination of ASCH Cytotoxicity to RAW264.7 cells

The cytotoxicities of mLFC cells treated with 200, 100, 50, 10, and 1  $\mu\text{g/ml}$  of ASCH were 83.5 $\pm$ 4.0, 88.3 $\pm$ 3.6, 91.8 $\pm$ 5.5, 94.9 $\pm$ 3.7, and 97.6 $\pm$ 4.8% of the control group respectively. Similar measurement of cytotoxicity in RAW264.7 cells by 200, 100, 50, 10, and 1  $\mu\text{g/ml}$  of ASCH treatments were 84.7 $\pm$ 3.9, 89.1 $\pm$ 3.3, 93.8 $\pm$ 5.0, 92.5 $\pm$ 4.1, and 96.2 $\pm$ 3.2 % of the control group (Table 1, Fig. 1). These data suggest that ASCH itself was not cytotoxic to cells used in the present study in 1 - 200  $\mu\text{g/ml}$  range of ASCH.

2. RT-PCR in RAW264.7 lines

1) Effects of ASCH treatment on IL-1 $\beta$  gene expression

Expression levels (in Ht values) of IL-1 $\beta$  mRNA determined by RT-PCR were 48 for untreated normal animal group, and 102 for saline control group. The Ht values for ASCH treatments were 62, 88, and 95 for 100, 10, and 1  $\mu\text{g/ml}$  of ASCH concentrations respectively. These data suggest that ASCH inhibited IL-1 $\beta$  mRNA expression in a concentration-dependent manner.

Table 1. Cytotoxicity of ASCH Extract Against Mouse Lung Fibroblast Cells and RAW264.7 Cell Line

Drug	Dose(mg/ml)	% of Control	
		mLFCs	RAW264.7
Control	0	100 $\pm$ 4.6	100 $\pm$ 4.3
	1	97.6 $\pm$ 4.8	96.2 $\pm$ 3.2
ASCH	10	94.9 $\pm$ 3.7	92.5 $\pm$ 4.1
	50	91.8 $\pm$ 5.5	93.8 $\pm$ 5.0
	100	88.3 $\pm$ 3.6	89.1 $\pm$ 3.3
	200	83.5 $\pm$ 4.0	84.7 $\pm$ 3.9

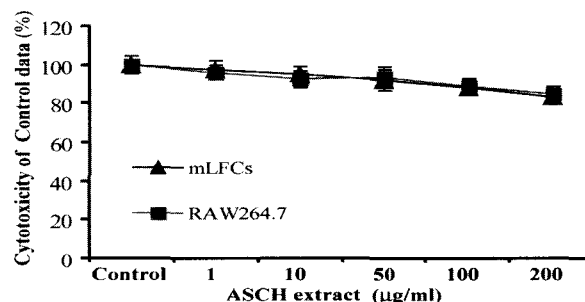


Fig. 1. Cytotoxicity effects of ASCH extract on mouse mLFCs and RAW264.7 cell line. Mouse lung fibroblast cells (mLFCs) and RAW264.7 cells were pretreated with various concentration ASCH extract. The results are expressed as the mean  $\pm$  S.E (N=6).

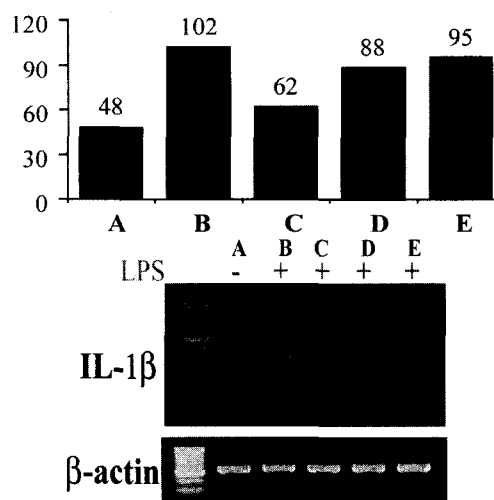


Fig. 2. Inhibitory effects of ASCH extract on IL-1 $\beta$  mRNA gene expression in RAW264.7 cell line. RAW264.7 cells were pretreated with various concentration of ASCH (100  $\mu\text{g/ml}$  (C), 10  $\mu\text{g/ml}$  (D), 1  $\mu\text{g/ml}$  (E)) extract in the presence (B) or absence (A) of lipopolysaccharide (LPS, 2  $\mu\text{g/ml}$ ) for 6 hr. Proinflammatory cytokine IL-1 $\beta$  mRNA expression was analyzed by RT-PCR and agarose gel electrophoresis, and quantitative data (Ht values) are shown in the Figure.

2) Effects of ASCH on IL-6 mRNA expression.

In RAW264.7 cells, levels of IL-6 mRNA expression determined by RT-PCR were 14 for untreated normal animal group and 96 for saline control group in terms of Ht values. The Ht values in ASCH treated group were 32, 94, and 95 for 100, 10, 1  $\mu\text{g}/\text{ml}$  of ASCH concentrations respectively (Fig. 3). These data suggest that ASCH inhibited IL-6 mRNA expression in a concentration-dependent manner.

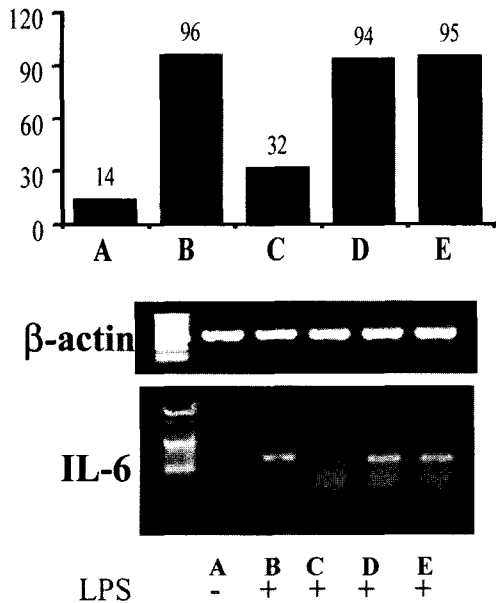


Fig. 3. Inhibitory effects of ASCH extract on IL-6 mRNA gene expression in RAW264.7 cell line. RAW264.7 cells were pretreated with various concentrations of ASCH extract [100  $\mu\text{g}/\text{ml}$  (C), 10  $\mu\text{g}/\text{ml}$  (D), and 1  $\mu\text{g}/\text{ml}$  (E)] in the presence (B) or absence of (A) lipopolysaccharide (2  $\mu\text{g}/\text{ml}$ ) for 6 hr. Expression of proinflammatory cytokines IL-6 mRNA in RAW264.7 cell line was analyzed by RT-PCR and agarose gel electrophoresis, and quantitative data are shown in the bar graph in the Figure.

3) Effects of IL-18 mRNA expression

In RAW264.7 cells, levels of IL-18 mRNA expression determined by RT-PCR were 35 for untreated normal animal group, and 101 for saline control group in terms of Ht values. The Ht values in ASCH treated group were 20, 127, and 131 for 100, 10, and 1  $\mu\text{g}/\text{ml}$  of ASCH concentrations respectively (Fig. 4). These data suggest that ASCH inhibited IL-18 mRNA expression in a concentration dependent manner.

4) Effects of ASCH on COX-2 mRNA expression

In RAW264.7 cells, levels of COX-2 mRNA expression determined by RT-PCR were 21 for untreated normal animal group and 205 for saline control group in terms of Ht values. The Ht values in ASCH treated group were 171, 198, and 203 for 100, 10, and 1  $\mu\text{g}/\text{ml}$  of ASCH concentrations respectively (Fig 5). These data suggest that ASCH inhibited COX-2 mRNA expression in a concentration dependent manner.

5) Effects of ASCH on NOS-II mRNA expression

In RAW264.7 cells, levels of NOS-II mRNA expression

determined by RT-PCR were 26 for untreated normal animal group and 199 for saline control group in terms of Ht values. The Ht values in ASCH treated group were 33, 178, and 185 for 100, 10, and 1  $\mu\text{g}/\text{ml}$  of ASCH concentrations respectively (Fig. 5). These data suggest that ASCH inhibited NOS-II mRNA expression in a concentration dependent manner.

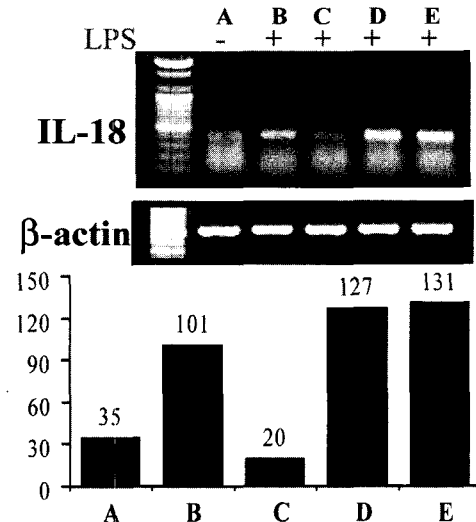


Fig. 4. Inhibitory effects of ASCH extract on IL-18 mRNA gene expression in RAW264.7 cell line. RAW264.7 cells were pretreated with various concentrations of ASCH extract [100  $\mu\text{g}/\text{ml}$  (C), 10  $\mu\text{g}/\text{ml}$  (D), 1  $\mu\text{g}/\text{ml}$  (E)] in the presence (B) or absence of (A) lipopolysaccharide (2  $\mu\text{g}/\text{ml}$ ) for 6 hr. Expression of proinflammatory cytokines IL-18 mRNA in RAW264.7 cell line was analyzed by RT-PCR and agarose gel electrophoresis, and quantitative data are shown in the bar graph in the Figure.

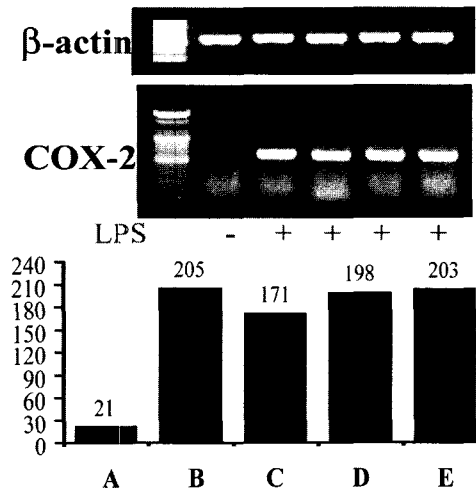


Fig. 5. Inhibitory effects of ASCH extract on COX-2 mRNA gene expression in RAW264.7 cell line. RAW264.7 cells were pretreated with various concentrations of ASCH [100  $\mu\text{g}/\text{ml}$  (C), 10  $\mu\text{g}/\text{ml}$  (D), 1 (E)  $\mu\text{g}/\text{ml}$ ] extract in the presence (B) or absence of (A) lipopolysaccharide (2  $\mu\text{g}/\text{ml}$ ) for 6 hr. Expression of pro-inflammatory cytokines COX-2 mRNA in RAW264.7 cell line was analyzed by RT-PCR and agarose gel electrophoresis and quantitative data are shown in the bar graph in the Figure.

6) Effects of ASCH on TNF- $\alpha$  mRNA expression

In RAW264.7 cells, levels of TNF- $\alpha$  mRNA expression determined by RT-PCR were 84 for untreated normal animal

group and 184 for saline control group in terms of Ht values. The Ht values in ASCH treated group were 190, 199, and 202 for 100, 10, and 1  $\mu\text{g}/\text{ml}$  of ASCH concentrations (Fig. 6). These data indicate that ASCH treatment did not change significantly TNF- $\alpha$  mRNA expression.

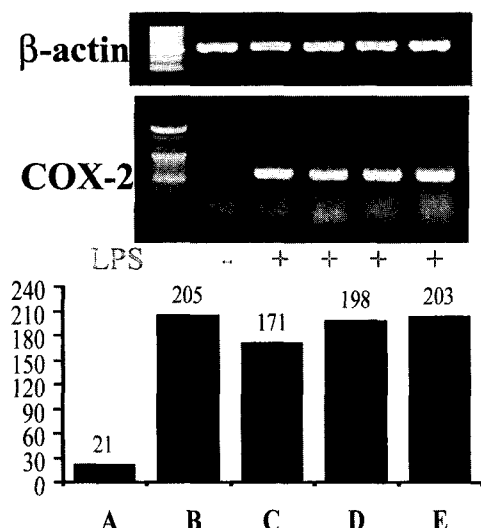


Fig. 6. Inhibitory effects of ASCH extract on NOS-II mRNA gene expression in RAW264.7 cell line. RAW264.7 cell line were pretreated with various concentrations of ASCH extract [100  $\mu\text{g}/\text{ml}$  (C), 10  $\mu\text{g}/\text{ml}$  (D), 1  $\mu\text{g}/\text{ml}$  (E)] in the presence (B) or absence (A) lipopolysaccharide (2  $\mu\text{g}/\text{ml}$ ) for 6 hr. Expression of pro-inflammatory cytokines NOS II mRNA in RAW264.7 cell line was analyzed by RT-PCR and agarose gel electrophoresis, and quantitative data are shown in the bar graph in the Figure.

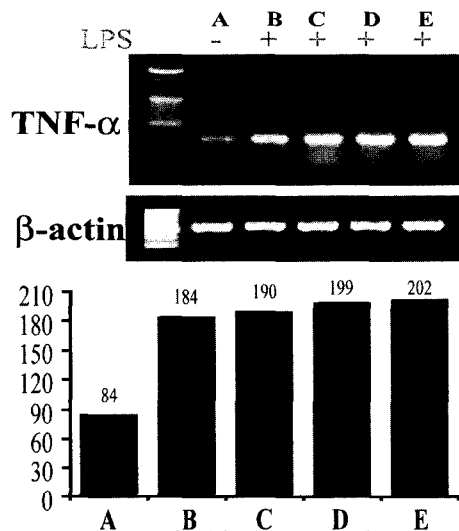


Fig. 7. Inhibitory effects of ASCH extract on TNF- $\alpha$  mRNA gene expression in RAW264.7 cell line. RAW264.7 cell line were pretreated with various concentrations of ASCH extract (100  $\mu\text{g}/\text{ml}$  (C), 10  $\mu\text{g}/\text{ml}$  (D), 1  $\mu\text{g}/\text{ml}$  (E)] in the presence (B) or absence (A) of lipopolysaccharide (2  $\mu\text{g}/\text{ml}$ ) for 6 hr. Expression of pro-inflammatory cytokines TNF- $\alpha$  mRNA in RAW264.7 cell line was analyzed by RT-PCR and agarose gel electrophoresis, and quantitative data are shown in the bar graph in the Figure.

### 3. Cytokine measurement by ELISA

#### 1) IL-6 production

In RAW264.7 cells, levels of IL-6 production were  $5.2 \pm 1.2$

ng/ml for untreated normal animal group and  $104.1 \pm 21.3$  ng/ml for saline control group. In ASCH treated group, IL-6 protein levels were  $47.1 \pm 13.0$ ,  $76.5 \pm 12.8$ , and  $88.5 \pm 21.1$  ng/ml for 100, 10 and 1  $\mu\text{g}/\text{ml}$  of ASCH respectively (Table 2, Fig. 8). These data indicate that ASCH treatment significantly decreased IL-6 production in RAW264.7 cells compared to saline treatment.

Table 2. Inhibitory Effect of ASCH Extract on the IL-6 Production in RAW264.7 Cell Line

Drug	ASCH ( $\mu\text{g}/\text{ml}$ )	IL-6 production (ng/ml)
Media control	0	$5.2 \pm 1.2$
L P S	Control	$104.1 \pm 21.3$
	100	$47.1 \pm 13.0^*$
	10	$76.5 \pm 12.8$
	1	$88.5 \pm 21.1$

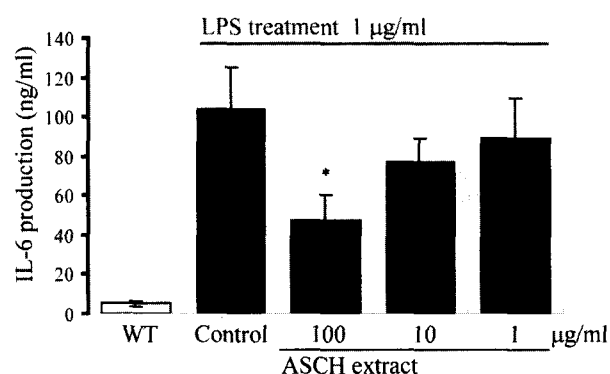


Fig. 8. Inhibitory effect of ASCH extract on the interleukin-6 production in RAW264.7 cell line. RAW264.7 cell line were pretreated with various concentrations of ASCH extract (100, 10, 1  $\mu\text{g}/\text{ml}$ ) in the presence or absence of lipopolysaccharide (1  $\mu\text{g}/\text{ml}$ ) for 24 hr. The culture supernatant was collected after 24 hr and IL-6 concentration in the supernatant was assayed by ELISA and other assay procedures as described in Materials and Methods. Data represent mean  $\pm$  S.E (N=3). Statistical comparison was made by t-test (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

#### 2) Effects of ASCH on TNF- $\alpha$ production in RAW264.7 cells

In RAW264.7 cells, levels of TNF- $\alpha$  production were  $12.5 \pm 4.3$  ng/ml for untreated normal animal group and  $86.8 \pm 16.6$  ng/ml for saline control group. In ASCH treated group, IL-6 protein levels were  $79.5 \pm 14.4$ ,  $85.3 \pm 13.7$ , and  $81.5 \pm 16.4$  ng/ml for 100, 10, and 1  $\mu\text{g}/\text{ml}$  of ASCH respectively (Table 3, Fig. 9). These data indicate that ASCH treatment decreased TNF- $\alpha$  production in a dose-dependent manner in RAW264.7 cells compared to saline treatment.

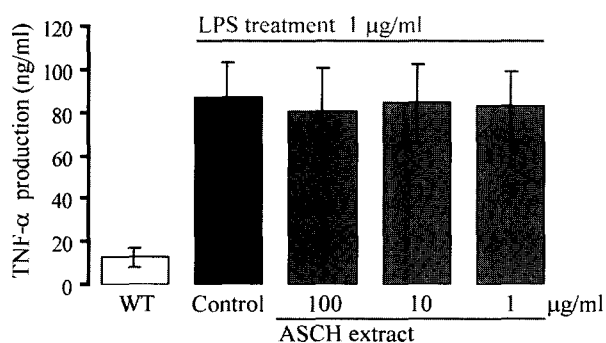
#### 4) Measurement of nitric oxide (NO) production in RAW264.7 cells

In RAW264.7 cells, levels of NO production were  $0.074 \pm 0.013$  for untreated normal animal group and  $0.546 \pm 0.082$  for saline control group. In ASCH-treated group, IL-6 protein levels were  $0.204 \pm 0.019$ ,  $0.311 \pm 0.037$  and  $0.479 \pm 0.044$  for 100, 10, and 1  $\mu\text{g}/\text{ml}$  of ASCH respectively (Table 4, Fig. 10). These data indicate that ASCH treatment significantly

decreased NO production in RAW264.7 cells compared to saline treatment.

**Table 3. Inhibitory Effect of ASCH Extract on the TNF- $\alpha$  Production in RAW264.7 Cell Line**

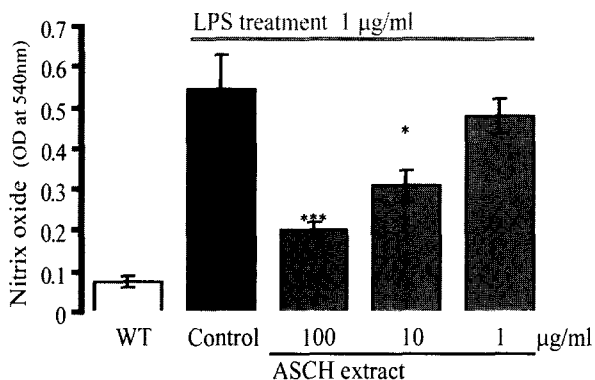
Drug	ASCH( $\mu$ g/ml)	TNF- $\alpha$ production (ng/ml)
Media control	0	5.2 $\pm$ 1.2
	Control	86.8 $\pm$ 16.6
L P S (1 mg/ml)	100	79.5 $\pm$ 14.4
	10	85.3 $\pm$ 13.7
	1	81.5 $\pm$ 16.4



**Fig. 9. Inhibitory effect of ASCH extract on the tumor necrosis factor- $\alpha$  production in RAW264.7 cell line.** RAW264.7 cells were pretreated with various concentration of ASCH extract (100, 10, 1  $\mu$ g/ml) in the presence or absence of lipopolysaccharide (1  $\mu$ g/ml) for 24 hr. The culture supernatant was collected 24 hr later and TNF- $\alpha$  concentration in the supernatant was assayed by ELISA and the other assay procedures as described in Materials and Methods. Data represent mean  $\pm$  S.E (N=3).

**Table 4. Inhibitory Effect of ASCH Extract on the Nitric Oxide Production in RAW264.7 Cell Line**

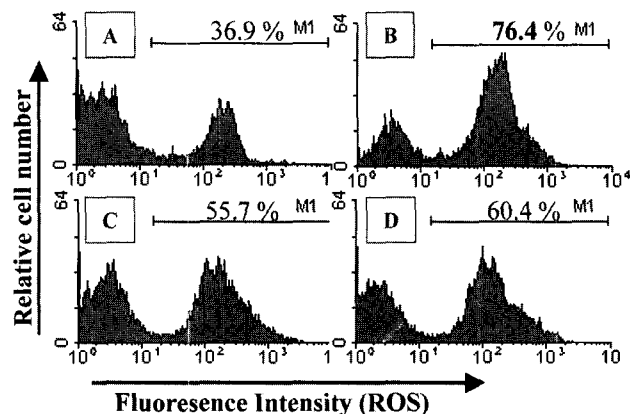
Drug	ASCH( $\mu$ g/ml)	Nitric oxide production (optical density, OD)
Media control	0	0.074 $\pm$ 0.013
	Control	0.546 $\pm$ 0.082
L P S (1 mg/ml)	100	0.204 $\pm$ 0.019***
	10	0.311 $\pm$ 0.037*
	1	0.479 $\pm$ 0.044



**Fig. 10. Inhibitory effect of ASCH extract on the nitric oxide (NO) release in RAW264.7 cell line.** RAW264.7 cells were pretreated with various concentrations of ASCH (100, 10, 1  $\mu$ g/ml) extract in the presence or absence of lipopolysaccharide (1  $\mu$ g/ml) for 48 hr. The culture supernatant was collected 24 hr later and NO concentration in the supernatant was determined as described in the Materials and Methods. Data represent mean  $\pm$  S.E (N=6). Statistical comparison was made by t-test (\* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001).

5) Analysis of reactive oxygen species (ROS) within the cell

Levels of ROS production was significantly increased in saline injected group compared to untreated control as shown in Fig 11. ROS levels were decreased in ASCH treated group in a dose dependent manner.



**Fig. 11. Inhibitory effect of ASCH extract on the ROS production in RAW264.7 cell line.** RAW264.7 cells were pretreated with various concentrations of ASCH extract (100  $\mu$ g/ml in C and 10  $\mu$ g/ml in D) in the presence (1  $\mu$ g/ml; B) or the absence (A) of LPS for 48 h. After culture, cells were treated with DCFH-DA reagent (50 mM), harvested, and used for the measurement of ROS levels (M1) by flow cytometry.

## Discussion

In the present study, ASCH, a herbal drug was examined whether it has anti-inflammatory effects in inflammation-induced macrophages. Analysis of major cytokines and inflammation-related enzymes at mRNA and proteins revealed significant decreases by ASCH treatment in macrophage cell lines tested. The present results suggest the physiological significance of ASCH in alleviating inflammation. ASCH in oriental medicine has been used to treat jaundice and diseases caused by gall stone formation. In particular, scoparon, chlorogenic acid, and caffeic acid of ASCH components are known to stimulate bile salts and hepatic protections from carbon tetrachloride (CCl<sub>4</sub>) toxicification. It is also believed that ASCH has antipyretic, anti-bacterial and anti-viral effects, and lowers blood pressure and blood cholesterol. In relation to anti-inflammatory reactions, ASCH was demonstrated to be effective for preventing edema caused by carrageenin.<sup>28,29</sup> In order to examine whether these previous reports on its anti-inflammatory effects are reproducible in the in vitro experimental model system, macrophage cell line RAW264.7 was used to investigate changes in inflammatory cytokines. Inflammation was induced by LPS treatment and induction of IL-1 $\beta$ , IL-6, IL-18, and TNF- $\alpha$  molecules were determined at protein levels by ELISA, or at mRNA levels by RT-PCR analysis. Significant increases in these cytokines were clearly

observed. Then, the treatment of ASCH decreased induction levels of inflammatory cytokines when measured at mRNA and protein levels though some variations in decreasing effects of ASCH were observed. For instance, TNF- $\alpha$  mRNA was not altered by increasing ASCH doses to 100  $\mu\text{g}/\text{ml}$  while protein levels were slightly decreased by 100  $\mu\text{g}/\text{ml}$  of ASCH treatment, suggesting that inhibitory effects of ASCH on the TNF- $\alpha$  production can be primarily regulated at post-translational level. Decreases in IL-1 $\beta$  and IL-6 protein and mRNA were observed, indicating inhibitory action of ASCH at the mRNA expression level. It is also noted that the inhibitory effects of ASCH were dose-dependent; in all cases, the highest dose used (100  $\mu\text{g}/\text{ml}$ ) was most effective in inhibiting cytokine production.

ASCH was also effective in inhibiting inflammation-related enzymes. COX-2 is an enzyme which converts arachidonic acid to prostaglandin.<sup>41,54</sup> Since synthesized prostaglandin itself and derived prostacyclin and thromboxanes are all involved in vasoconstriction or vasodilation, edema and other inflammation-related responses, regulation of COX-2 (and also COX-1) is very important for the development of inflammation regulation.<sup>25,30</sup> For instance, classical COX inhibitor aspirin and indomethacin are good examples as anti-inflammatory drugs. The present RT-PCR analysis data showed that COX-2 mRNA levels were strongly induced in macrophages by 2  $\mu\text{g}/\text{ml}$  of LPS treatment and then only slightly decreased at 100  $\mu\text{g}/\text{ml}$  of ASCH. With these results, it is hard to evaluate whether ASCH is effective for COX-2 inhibition until further studies on COX-2 protein and enzyme activity determination are performed. Also important is to determine the regulation of COX-1 expression since this enzyme is equally important to produce prostaglandin in many tissues including gastric mucosa where COX-2 is known to be induced. Changes in NOS-II mRNA expression were investigated in a similar procedure in macrophage cells in the presence or the absence of ASCH. We found that ASCH at high concentration (100  $\mu\text{g}/\text{ml}$ ) inhibited very efficiently NOS-II mRNA synthesis, which was virtually to the level of untreated macrophages (Fig. 6).

Although the levels of NOS-II protein were not determined, measurement of NO production showed a significant decrease by ASCH treatment in a dose-dependent manner. Thus, ASCH appears to be more efficient for inhibiting NOS-II activity compared to COX-2 activity.

These data suggest that cells exposed to noxious stimulation causing injury and inflammation could be protected by ASCH treatment. NO is synthesized by endothelial cells (mostly via type III) NO synthase (eNOS) and by macrophages (mostly via type II) NO synthase (iNOS).<sup>8,44,45,48</sup> NO, whatever the sources are, causes vasodilation and reduces platelet and

leukocytes adhesion; NO free radicals are also cytotoxic to microbial and mammalian cells.<sup>10,48</sup>

In addition to NO, reactive oxygen species are important for inducing diverse chemical reactions within the cells causing cell damage.<sup>15,47</sup> Our data further showed reduction in the production of reactive oxygen species. Although our analysis methods cannot explain which reactive molecules are specifically inhibited, overall measurement of ROS using DCFH-DA reagent indicates significant decreases in ROS by ASCH treatment in a dose-dependent manner. Certain unknown components in the ASCH could have antioxidant actions, induce the production of antioxidants in the cells, or induce enzymes such as SOD and catalase which are involved in the degradation pathway of reactive oxygens. Recently, scientific concerns have been growing to understand mechanisms underlying the effectiveness of the herbal drugs for the treatment of diverse diseases including inflammatory disease, immune diseases and cancer.<sup>13,17,31,33,42,51,50</sup> In the present study, we have found that ASCH plays a role in alleviating inflammatory response in cultured macrophage cells, as has been similarly demonstrated in other kind of herbal drugs.<sup>17,31,33</sup> Yet, it would be important to determine time-dependent changes in the production of inflammatory cytokines. Inflammation response by certain levels are the host protective response intended to eliminate the initial cause of cell injury as well as the necrotic cells and tissues resulting from the original insults. Inflammation accomplishes its protective mission by diluting, destroying or otherwise neutralizing harmful agents (e.g., microbes or toxins). It then sets into the motion the events that eventually heal and reconstruct the sites of injury. In general, early stages of inflammatory event are important as being protective whereas prolonged inflammation could be harmful to the cells. In the present study, cells were exposed to ASCH for 1 hr and then 48 hr with LPS, which can be regarded as a long treatment. Further studies in which cells are exposed for a shorter time period would provide the responsiveness of cells and production pattern of inflammatory cytokines. Our data showed that ASCH treatment to normal RAW264.7 cells did not produce any cytotoxicity. Thus, time-dependent investigation of ASCH treatment in inflammation-induced macrophage cell lines may provide insight to understand mechanisms on anti-inflammation mechanisms.

## Conclusion

The present study was performed to examine the possible anti-inflammatory actions of a herbal drug ASCH in RAW264.7



cell line. Inflammation was induced by LPS toxin, and measurement of inflammatory responses in terms of induction of inflammatory cytokines and inflammation-related enzymes were increased and then treatment of ASCH decreased these chemical mediators. While these data implicate its usefulness as a therapeutic agents against harmful inflammation, further studies are needed especially in relation to specific pathological inflammatory disease. Also, time- dependent changes in the responsiveness of chemical mediators need to be carefully examined and cytotoxicity in parallel should be determined. Also, possible synergistic or antagonistic actions by examining together with other herbal drugs may provide useful information for the elucidation of specific components (e.g., antioxidant or its inducers in the herbal drugs). The evidence-based studies then provide the basis of useful drug design for the clinical applications aiming for the numerous inflammatory diseases.

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