Original Article

The Acupuncture Vol. 34 No. 1 February 2017 : 11-21
pISSN 1229-1137 eISSN 2287-7797
http://dx.doi.org/10.13045/acupunct.2017070

MOK, a Pharmacopuncture Medicine, Reduces Inflammatory Response through Inhibiting the Proinflammatory Cytokine Production in LPS-stimulated Mouse Peritoneal Macrophages※

Ji Hye Hwang[1], Min Sub Hwang[2], Yong-ki Park[1,3,*]

[1]Korean Medicine R&D Center, Dongguk University
[2]Dept. of Acupuncture & Moxibustion Medicine, College of Korean Medicine, Dongguk University
[3]Department of Herbology, College of Korean Medicine, Dongguk University

[Abstract]

Objectives : In this study, we investigated the anti-inflammatory and anti-oxidative effects of MOK, a pharmacopuncture medicine, in lipopolysaccharide (LPS)-stimulated mouse peritoneal macrophages.

Methods : Peritoneal macrophages were isolated from ICR mice. Primary macrophages were treated with MOK extract (1.25, 2.5, 5, 10, and 20 mg/ml) for 30 min and then stimulated with LPS (1 μg/ml) for the indicated times. Cytotoxicity was measured using MTT and LDH assays. Nitric oxide (NO) production in culture supernatants was measured using the Griess assay. The mRNA expression of iNOS, COX-2, proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and antioxidant enzymes (HO-1 and MnSOD) was measured by RT-PCR.

Results : Treatment with MOK extract (2.5, 5, and 10 mg/ml) significantly decreased LPS-induced NO production in peritoneal macrophages through inhibition of iNOS expression. The expression of COX-2, TNF-α, IL-1β, and IL-6 mRNA was also decreased in LPS-stimulated macrophages upon treatment with MOK extract. MOK treatment also increased the expression of HO-1 and MnSOD mRNA in macrophages.

Conclusion : These results indicate that MOK exerts anti-inflammatory and antioxidant effects by regulating the transcription levels of inflammatory mediators and antioxidant proteins in activated macrophages.

Key words :
Antiinflammation;
Antioxidation;
LPS;
MOK;
Peritoneal macrophages;
Pharmacopuncture

Received : 2017. 01. 11.
Revised : 2017. 02. 02.
Accepted : 2017. 02. 06.
On-line : 2017. 02. 20.

※ This research was supported by the Korean Health Technology R&D Project through the Korean Health Industry Development Institute (KHIDI) funded by the Ministry of Health& Welfare, Republic of Korea (grant number : HI16C0622).

* Corresponding author : Department of Herbology, College of Korean Medicine, Dongguk University, Dongdaero 123, Gyeongju 38066, Republic of Korea
Tel : +82-54-770-2661 E-mail : yongki@dongguk.ac.kr

Ⅰ. Introduction

Pharmacopuncture therapy is a new stimulating method for acupoints, and involves injection of herbal extract at acupoints. This method has been frequently used for the regulation of immune balance in the clinical setting[1,2].

MOK is a medicinal extract with ten different herbs for pharmacopuncture therapy in Korean clinics, and is modified from OK extract, which is commonly used for pharmacopuncture treatment of patients with thyroid syndromes such as hypothyroidism and hyperthyroidism. MOK consists of OK which is known to have anti-inflammatory effects[1-3] and Hominis Placenta, and MOK is also applied clinically in heart disease and Hwa-Byung[2-5].

Among the constituents of MOK, Moschus (*Moschus berezovskii*), Bovis Calculus (*Bos taurus*), Ursi Fel (*Ursus arctos*) and Hominis Placenta are the main components. Moschus is a representative orifice-opening medicine and recently, it has been widely used as a constituent of pharmacopuncture besides an internal medicine in clinical practice. It has also been reported to have hepatoprotective activity, memory improvement effect, and anti-cancer activity. Ursi Fel and Bovis Calculus are representative heat-clearing medicines. Ursi Fel has been reported to possess hepatoprotective and anti-inflammatory activities, and Bovis Calculus has been reported to improve cerebral blood flow, systemic blood pressure, and lipid metabolism[3,6,7]. Hominis Placenta, a tonifying and replenishing medicine, has the capacity to tonify qi, blood, and essence, and is used for treatment in conditions requiring tissue regeneration, regulation of immune response, prevention of infection, promotion of hormone function and improvement of dysmenorrhea, climacteric symptoms, peripheral facial paralysis, osteoporosis, arthritis, and so on[6,8].

However, although MOK is widely used in Korean clinics for the treatment of heart and thyroid diseases, little scientific evidence is available for its activity. In this study, we investigated the effects of MOK on inflammatory and oxidant responses using activation of mouse peritoneal macrophages.

Ⅱ. Materials and Methods

1. Materials

1) Preparation of MOK extract

MOK extract was provided by the Korea Immuno-Pharmacopuncture Association (Seoul, Korea) and was manufactured under a Good Manufacturing Practice (GMP) compliant facility (Korea Immuno-Pharmacopuncture Association, Seoul, Korea). The quality of all the raw materials in MOK was approved by the Korea Food & Drug Administration (KFDA). MOK was extracted with dried herbs (106.2 g) in distilled water (1 L) for 3 hours and then mixed with alcohol to 1:1, filtered through a two-layer mesh and then concentrated under vacuum pressure. After freeze-dried MOK was dissolved in water (53.1 mg/ml) (Table 1).

2) Reagents

Lipopolysaccharide (LPS, L2630), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetraolium bromide (MTT), sulfanilamide, and N-1-napthylethylenediamine dihydrochloride (NED) were purchased from Sigma-Aldrich (St Louis, MO, USA). RPMI1640, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from GenDEPOT (Barker, TX, USA). TRIzol reagent was obtained from Bio-Rad Laboratories (Philadelphia, PA, USA). M-MLV reverse transcriptase, and lactate dehydrogenase (LDH) Cytotoxicity Assay Kit were obtained from Promega (Madison, WI, USA). Taq-based PCR enzyme was purchased from Toyobo (Osaka, Japan). Thioglycollate broth was purchased from Difco Laboratories (Detroit, MI, USA).

3) Animals

Male ICR mice (5-weeks old, 17-21 g) were obtained from Orient Bio Inc. (Gyeonggi-do, Korea). The animals were housed under controlled environmental conditions with an ambient temperature of 23±1℃, a relative humidity of 50±10%, a 12 h light/dark cycle, and free access to food and water.

2. Methods

1) Isolation and culture of peritoneal macrophages

Peritoneal exudates were obtained from male ICR mice by lavage after 4 days of intraperitoneal injection with 2 ml of 3% thioglycollate broth as reported previously[9]. After washing with RPMI-1640 medium containing 2% FBS, 100 U/ml penicillin, and 100 ㎍/ml streptomycin, the peritoneal macrophages (1×10^6 cells/ml) were plated in culture dishes for 4 h at 37℃ in a 5% CO_2 incubator. After discarding the unattached cells, primary macrophages were maintained in RPMI-1640 medium containing 10% FBS at 37℃ in a 5% CO_2 incubator.

2) MTT assay

To determine the toxic range of MOK extract in peritoneal macrophages, cell viability was measured using an MTT-based colorimetric assay. Briefly, peritoneal macrophages (5×10^4 cells/well) were seeded in 96-well plates and treated with MOK extract at different concentrations for 24 h at 37℃ in a 5% CO_2 incubator. Upon culture termination, 10 ㎕ of MTT solution (5 mg/ml) was added to each well, and incubated for 4 h. The resulting crystals were dissolved in 100 ㎕ DMSO, and absorbance at 570 nm was measured using a microplate reader (GENios, TEKAN Instruments, Inc., Austria). Cell viability was calculated by the formula: (viable cells)% = (OD of MOK-treated sample/OD of untreated sample) × 100

3) LDH assay

LDH is a well-known marker of cell membrane integrity and cell viability, and its accumulation is the result of plasma membrane breakdown and altered permeability at the stage of secondary necrosis in the late stage of apoptosis[10]. Therefore, we next measured the cytotoxic response to MOK extract, by measuring the release of LDH leakage in cell culture medium according to the manufacturer's instructions. Briefly, peritoneal macrophages (5×10^4 cells/well) were seeded in 96-well plates and treated with MOK extract at different concentrations for 24 h at 37℃ in a 5% CO_2 incubator. CytoTox 96 Reagent (50 ㎕) was then added to each well, and the plate was covered with foil and incubated for 30 min at room temperature; 50 ㎕ of Stop Solution was then added to each well, and optical density (OD) at 490 nm was measured using a microplate reader (GENios, TEKAN Instruments, Inc., Austria). Cytotoxity was calculated by the formula: (Cytotoxity)% = (OD of MOK-treated sample/OD of Control sample (Maximum LDH Release)

4) NO assay

To measure the NO levels, peritoneal macrophages (1×10^5 cells/ml) were treated with MOK extract at different concentrations for 30 min, and then stimulated with LPS (1 ㎍/ml) or without it for 24 h at 37℃ in a 5% CO_2 incubator. NO levels were measured in culture supernatants by the Griess assay. Therefore, the culture supernatants (100 ㎕) were added to a 96-well plate, followed by 1% sulfanilamide (50 ㎕) and 0.1% NED (50 ㎕). The plate was then incubated for 15 min at room temperature in the dark, and the absorbance was measured at 540 nm in a microplate reader (GENios). A standard curve was generated in the same fashion using $NaNO_2$.

5) Reverse Transcription (RT)-PCR

Peritoneal macrophages were treated with MOK extract at different concentrations for 30 min, and then stimulated with LPS or without for 5 h at 37℃ in a 5% CO_2 incubator. Total RNA was isolated

from each cell using TRIzol reagent, and cDNA synthesis from total RNA with a mixture including oligo-dT primer, 5x RT buffer (Promega Co., Madison, WI, USA), 0.5 mM dNTP, 3 mM $MgCl_2$, RNase inhibitor, and Improm-II™ reverse transcriptase (2U) was carried out at 25℃ for 5 min and 42℃ for 60 min. The reaction was terminated at 70℃ for 10 min. PCR was carried out using specific primers for the target genes (Table 2), and PCR mixture [2 μl cDNA, 4 μM 5′ and 3′ primers, 10 x buffer (10 mM Tris-HCl, pH 8.3), 50 mM KCl, 0.1 % Triton X-100, 25 mM $MgCl_2$, 250 μM dNTPs, and 1 U Taq polymerase under the following incubation conditions: 30 s denaturation at 94℃, 30 s annealing at 58-60℃, 1 min extension, and 10 min final extension at the end of 30 cycles. Band intensity was quantified by automated densitometric analysis (ChemiDoc MP Imaging System (BioRad Laboratories, CA, USA).

Table 1. Constituents of MOK extract

Herbal name	Scientific name	Ratio (mg/ml)
Hominis Placenta	*Hominis Placenta*	2
Moschus	*Moschus berezovskii*	0.5
Fel Ursi	*Ursus arctos*	0.3
Calculus Bovis Cow bezoar	*Bos taurus*	0.3
Scutellariae Radix	*Scutellaria baicalensis*	10
Phellodendri Cortex	*Phellodendron amurense*	10
Pulsatilla Koreana	*Pulsatilla koreana*	10
Sophorae Subprostratae Radix	*Sophora tonkinensis*	10
Aucklandiae Radix	*Aucklandia lappa*	5
Aquilaria agallocha	*Aquilaria agallocha*	5

Table 2. Primer sequences for PCR

Name		Sequence (5′→3′)
iNOS	Forward	CCCGAAGTTTCTGGCAGC
	Reverse	GGCTGTCAGAGCCTCGTGGCTT
COX-2	Forward	GGAGAGACTATCAAGATAGTGATC
	Reverse	ATGTCAGTAGACTTTTACAGCTC
TNF-α	Forward	ATAGCTCCCAGAAAAGCAAGC
	Reverse	CACCCCGAAGTTCAGTAGACA
IL-1β	Forward	GCCTTGGGCCTCAAAGGAAAGAATC
	Reverse	GGAAGACACAGATTCCATGGTGAAG
IL-6	Forward	TGGAGTCACAGAAGGAGTGGCTAAG
	Reverse	TCTGACCACAGTGAGGAATGTCCAC
MnSOD	Forward	GTGACTTTGGGTCTTTTGAG
	Reverse	GCTAACATTCTCCCAGTTGA
HO-1	Forward	AAGATTGCCCAGAAAGCCCTGGAC
	Reverse	AACTGTCGCCACCAGAAAGCTGAG
GAPDH	Forward	GACATCATACTTGGCAGGTT
	Reverse	CTCGTGGAGTCTACTGGTGT

6) Statistical Analysis

GraphPad Prism (GraphPad Software, Inc., San Diego) was used for statistical analysis. Data were expressed as mean ± SEM (standard error of mean) of three independent experiments and were analyzed for statistical significance using analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons. Null hypotheses of no difference were rejected if *p*-values were less than 0.05.

Ⅲ. Results

1. Effects of MOK extract on cell viability and toxicity in peritoneal macrophages

To investigate the cytotoxic effects of MOK extract in peritoneal macrophages, we measured cell viability using both the MTT and LDH assays. MOK extract did not show any effect on cell viability (Fig. 1A) or cell toxicity (Fig. 1B) in a range of 1.25 to 20 mg/ml in peritoneal macrophages; however, color interference was observed at 20 mg/ml. Therefore, we used the MOK extract within 10 mg/ml for efficacy in the study.

2. Effects of MOK extract on NO production and iNOS mRNA expression in LPS-stimulated peritoneal macrophages

To investigate the inhibitory effects of MOK extract on inflammatory responses in activated macrophages, we measured NO production and iNOS expression in LPS-stimulated peritoneal macrophages by the Griess assay and RT-PCR, respectively. As shown in Fig. 2A, NO production was increased after LPS stimulation of macrophages, and treatment with MOK extract at 2.5, 5, and 10 mg/ml in LPS-simulated cells resulted in significantly reduced NO production in a concentration-dependent manner. In addition, MOK extract at 5 and 10 mg/ml significantly inhibited iNOS mRNA expression in LPS-stimulated macrophages (Fig. 2B).

3. Effects of MOK extract on COX-2 mRNA expression in LPS-stimulated peritoneal macrophages

We investigated the inhibitory effects of MOK extract on the expression of the inflammatory mediator, COX-2, in LPS-stimulated peritoneal macrophages by RT-PCR. As shown in Fig. 3, COX-2 mRNA expression was increased upon LPS

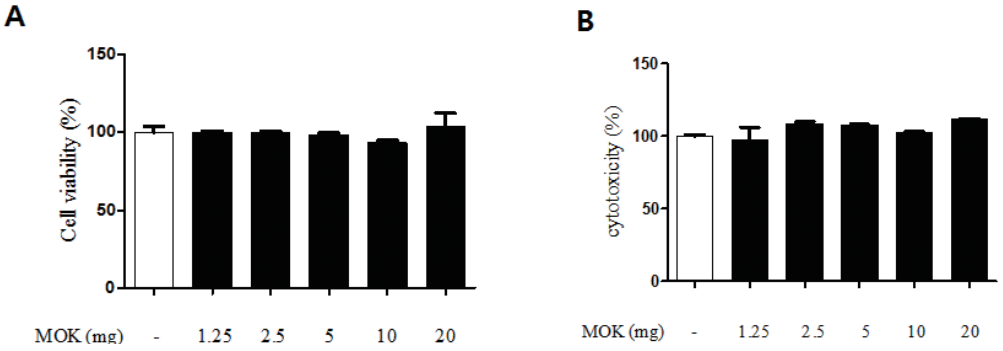

Fig. 1. Effects of MOK extract on cell viability and cytotoxicity in peritoneal macrophages. Cells were treated with MOK extract at 1.25, 2.5, 5, 10, and 20 mg/ml for 24 h. Cell viability was measured by the MTT assay, and cytotoxicity was measured by the LDH release assay. Data are mean ± SEM of three independent experiments

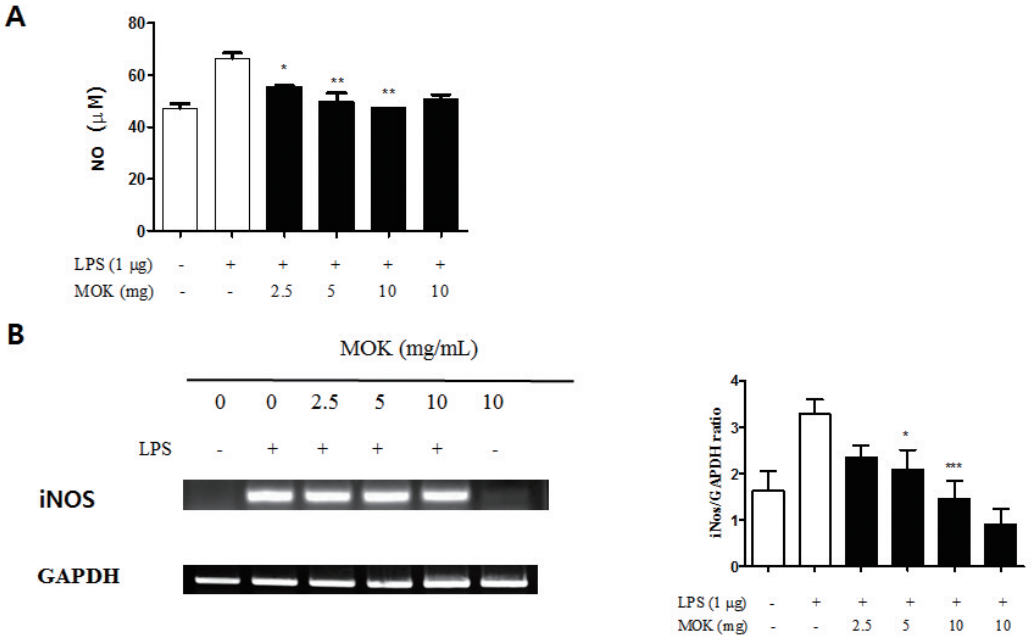

Fig. 2. Effect of MOK extract on NO production and iNOS mRNA expression in LPS-stimulated peritoneal macrophages. The cells were treated with MOK extract at 2.5, 5 and 10 mg/ml for 30 min, and then stimulated with or without LPS (1 μg/ml) for 5 h. (A) NO levels were measured in culture medium by the Griess assay. The expression of iNOS mRNA was determined by RT-PCR. GAPDH was used as an internal control. iNOS density was analyzed using GAPDH as the internal control, and represented as a histogram with mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. LPS alone

stimulation in peritoneal macrophages. Treatment with the MOK extract at 5 and 10 mg/ml significantly decreased the LPS-induced COX-2 expression (Fig. 3).

4. Effects of MOK extract on mRNA expression of pro-inflammatory cytokines in peritoneal macrophages

To better understand the inhibitory effect of MOK extract on the inflammatory responses in activated macrophages, mRNA expression of proinflammatory cytokines was investigated in LPS-stimulated peritoneal macrophages by RT-PCR. Treatment with MOK extract at 5 and 10 mg/ml significantly inhibited the expression of IL-1β, IL-6, and TNF-α mRNA in LPS-stimulated peritoneal macrophages (Fig. 4).

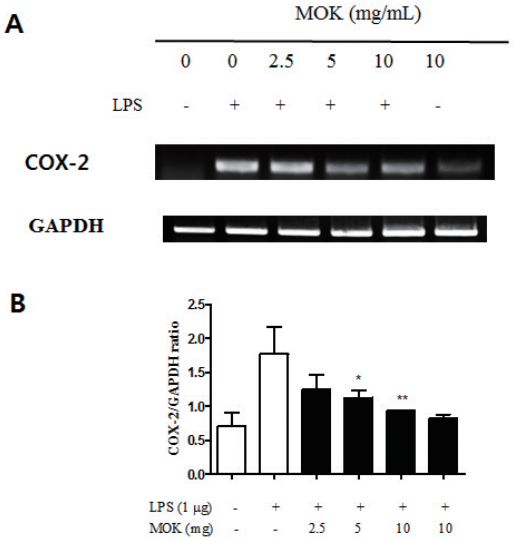

Fig. 3. Effect of MOK extract on COX-2 mRNA expression in LPS-stimulated peritoneal macrophages. The cells were treated with MOK extract at 2.5, 5, and 10 mg/ml for 30 min and then stimulated with LPS (1 μg/ml) or without for 5 h. (A) COX-2 expression was analyzed by RT-PCR. GAPDH was used as an internal control. (B) COX-2 density was analyzed using GAPDH as an internal control, and represented as a histogram with a mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. LPS alone

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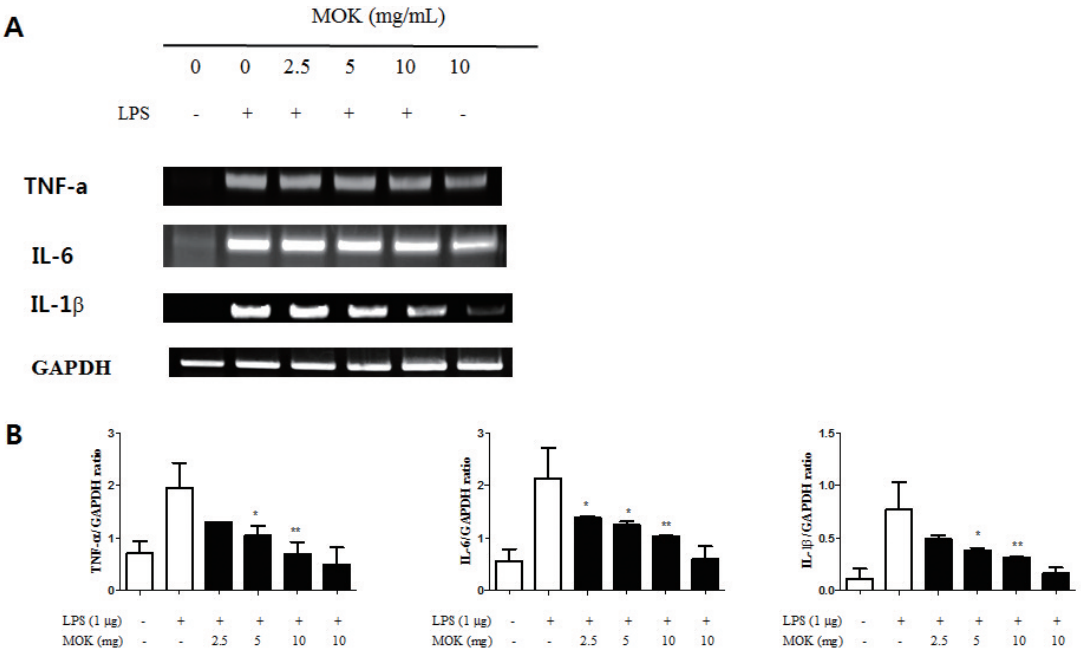

Fig. 4. Effect of MOK extract on the expression of proinflammatory cytokine mRNAs in LPS-stimulated peritoneal macrophages. The cells were treated with MOK extract at 2.5, 5, and 10 mg/ml for 30 min, and then stimulated with or without LPS (1 μg/ml) for 5 h. (A) The expression of TNF-α, IL-1β, and IL-6 mRNA was analyzed by RT-PCR. GAPDH was used as an internal control. (B) The density of each cytokine was analyzed using GAPDH as an internal control, and represented as a histogram with mean ± SD of three independent experiments. *$p < 0.05$, and **$p < 0.01$ vs. LPS alone

5. Effects of MOK extract on oxidative stress in LPS-stimulated peritoneal macrophages

To investigate the antioxidant effects of MOK extract in activated macrophages, we analyzed the mRNA expression of antioxidant enzymes in peritoneal macrophages by RT-PCR. The expression of HO-1 and MnSOD mRNA was significantly increased by treatment with the MOK extract at 10 mg/ml in peritoneal macrophages (Fig. 5).

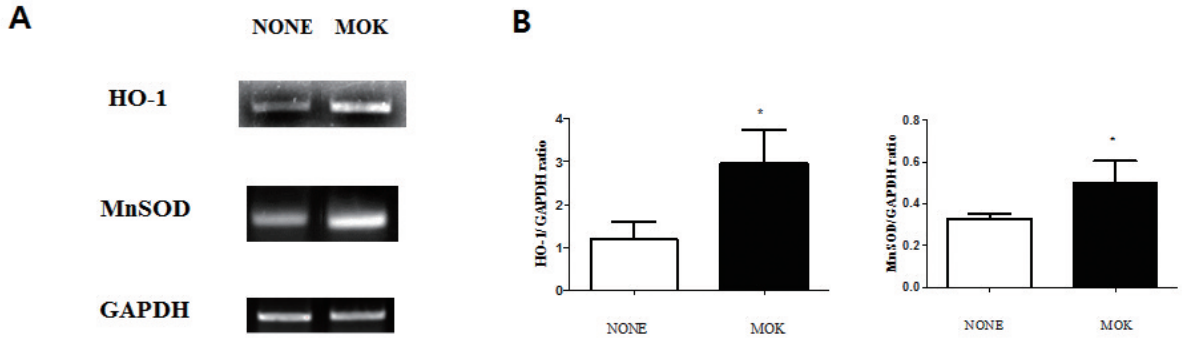

Fig. 5. Effect of MOK extract on the expression of HO-1 and MnSOD mRNA in peritoneal macrophages. The cells were treated with MOK extract at 10 mg/ml for 30 min, and (A) the expression of HO-1 and MnSOD mRNA was analyzed by RT-PCR. (B) The density of each cytokine was analyzed using GAPDH as an internal control, and represented as a histogram with mean ± SD of three independent experiments. *$p < 0.05$ vs. cells alone

Ⅳ. Discussion

Pharmacopuncture is a new method of acupuncture treatment in Traditional Korean Medicine (TKM). This method is widely used in clinics because it does not involve the digestive system, and can therefore work faster, with more efficacy than medicines administered orally[1,2]. In particular, Immuno-Pharmacopuncture therapy was first developed by Nam Sang-cheon and has been used with many extracts such as HN, I, OK, MOK, V, CO, CS, CA, CH, and S in clinical practice[2]. Among these, MOK consists of ten components: Hominis Placenta, Moschus, Fel Ursi, Calculus Bovis, Scutellariae Radix, Phellodendri Cortex, Pulsatilla Koreana, Sophorae Subprostratae Radix, Saussurea lappa, and Aquilaria agallocha. It is clinically used to treat for the meridian of fire in nature and symptoms related to heart disease such as fright palpitations, fearful throbbing, sleep disorders, angina pectoris, and thyroid diseases such as thyroid hyperthyroidism, hypothyroidism, thyroid hypertrophy, and goiter[2,4].

Immuno-Pharmacopuncture therapy with MOK is frequently administered in clinics, but little research[4,5] exists about its effects and the mechanism of action with its constituents. Moreover, there is no scientific evidence for MOK in which Hominis Placenta is newly added since 2003. To the best of our knowledge, this is the first efficacy study for evaluating the effect of MOK on the regulation of inflammatory responses in activated macrophages. This study will be helpful for the use of MOK in clinics.

MOK is a polyherbal extract for pharmacopuncture consisting of ten different constituents: Moschus is a representative orifice-opening medicine, and is a detoxification agent for treating inflammation, relieving swelling, and killing pain. It has been described as a highly valued ingredient in Chinese medicinal remedies[6,11]. Bovis Calculus is a heat-clearing and an orifice-opening medicine. Ursi Fel, Scutellariae Radix, Phellodendri Cortex, Pulsatilla Koreana, and Sophorae Subprostratae Radix are heat-clearing medicines. Aucklandiae Radix and Aquilaria agallocha are qi-regulating medicines. Hominis Placenta is a tonifying and replenishing medicine. Ursi Fel was reported to possess various properties such as antimicrobial, anti-inflammatory, anti-hepatotoxic, choleretic lithagogic, anti-liver fibrosis, anti-cancer, antipyretic and sedative, anticonvulsant and analgesic, hypotensive, anti-tussive and anti-asthmatic, and anti-stress and relaxing effects in modern pharmacological research[6,12]. *Bovis calculus* has been reported to exhibit antispasmodic, fever-reducing, anti-inflammatory, antioxidant and gallbladder-repairing effects[13]. Hominis placenta is a rich source of various bioactive substances, including peptides, nucleic acids, fatty acids, amino acids, enzymes, minerals, and trace elements[14,15] and is known to have various pharmacological effects such as anti-oxidation, anti-inflammation, and regeneration, especially acceleration of wound healing[12,16,17]. However, a comparison of the biological activity of MOK with those of single medicines has not been reported. In this study, we found that MOK has an anti-inflammatory effect in activated macrophages via downregulation of the inflammatory mediator transcription, and antioxidant effects through an increase in antioxidant enzyme expression.

Biological experiments are most often performed with immortalized cell lines because they are readily available and can be expanded without limitation. However, cell lines may differ from the in vivo situation in important aspects[18]. Therefore, in our study, we investigated the effects of MOK on the inflammatory response and oxidative stress in primary macrophages isolated from the peritoneal cavity of mice. Macrophages are major cells in the innate immune system, which act as the first line of defense against invading pathogens such as bacteria, viruses, and fungi and respond to their attack by releasing cellular signaling molecules and anti-microbial agents[19,20]. In the normal state, inflammatory mediators such as NO/iNOS, COX-2,

and proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 generated from macrophages play an essential role in host survival and tissue repair[21]. However, overproduction of these inflammatory mediators is closely related to the induction and progression of inflammatory diseases[22,23].

In response to proinflammatory stimulation with LPS, upregulation of iNOS occurs, releasing NO, a reactive free radical. High levels of NO produced by iNOS are cytotoxic to cells undergoing inflammation[24]. COX-2 also plays an important role in inflammation mediated by activated macrophages through the production of prostaglandin (PG) E_2 which is a major indicator of inflammation as well as NO[25]. In this study, we found that treatment with MOK extract in peritoneal macrophages effectively inhibited LPS-induced overproduction of NO through downregulation of iNOS expression and inhibition of COX-2 expression. This indicates that MOK extract has an anti-inflammatory effect in activated macrophages through inhibition of inflammatory mediator production.

Many biological molecules, including reactive oxygen species (ROS), NO, PGE2, and proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 are involved in the development of inflammation[23,26]. Macrophages are important producers of proinflammatory cytokines when they are exposed to stimuli such as LPS exposure[27]. These proinflammatory cytokines allow an increase in blood flow and capillary permeability, leading to infiltration of immune cells. TNF-α plays a central role in inflammatory and destructive processes commonly found in several human autoimmune and chronic inflammatory diseases[28]. IL-1β is also a major proinflammatory cytokine, and is important for the initiation and enhancement of inflammatory responses to the proliferation of some microorganisms[29]. IL-6 is an endogenous mediator of LPS-induced fever[30]. In the present study, MOK treatment significantly inhibited the expression of TNF-α, IL-1β, and IL-6 mRNA in LPS-stimulated peritoneal macrophages, suggesting that the MOK extract can decrease the production of these cytokines as well as that of iNOS and COX-2.

Oxidative stress induced by the overproduction of ROS that damage cellular lipids, proteins, and DNA is thought to be implicated in a variety of diseases or conditions, including some cancers, cardiovascular disease, acute inflammatory problems, diabetes mellitus complications, chronic inflammatory diseases such as rheumatoid arthritis and lupus erythematosus, photo-oxidative stress to the eye such as cataract, central nervous system disorders such as certain forms of familial amyotrophic lateral sclerosis, neurodegenerative disorders such as Parkinson's disease and Alzheimer's dementia, and age-related disorders, perhaps even including factors underlying the aging process itself[31,32]. The antioxidant system includes exogenous and endogenous components; the latter include antioxidant enzymes such as superoxide dismutases (CuZnSOD and MnSOD), glutathione peroxidase (GSPx), and heme oxygenase-1 (HO-1)[33]. The antecedent upregulation of HO-1 is necessary and sufficient for subsequent induction of the MnSOD gene in astroglia. The mechanism mediating MnSOD expression in astroglia overexpressing HO-1 is likely oxidative in nature[34].

In this study, MOK treatment induced the upregulation of MnSOD and HO-1 mRNA in peritoneal macrophages. This indicates that MOK extract has antioxidant potential.

We expect that MOK extract has various effects on the modulation of immune responses and protection of hepatic damage. In future studies, we will investigate other effects of MOK and on signaling pathways such as MAPKs and NF-kB that are regulated by inflammatory responses.

V. Conclusions

In summary, we demonstrated that MOK, an herbal mixture used in Immuno-Pharmacopuncture, can suppress LPS-induced inflammatory re-

sponses in peritoneal macrophages through inhibition of NO production, suppression of inflammatory mediators (iNOS, and COX-2), pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) mRNA expression, and an increase in antioxidant enzymes (HO-1 and MnSOD). These results suggest that MOK can be used as a safe anti-inflammatory treatment in pharmacopuncture.

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