Effects of MOK, a pharmacopuncture medicine, on the TH1/TH2 immune response and antioxidation in Con A–stimulated primary mouse splenocytes

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[Abstract]

Objectives: In this study, we investigated the immunomodulatory and antioxidant effect of MOK, a pharmacopuncture medicine, in concanavalin A (Con A)–stimulated mouse splenocytes.

Methods: Primary splenocytes were isolated from ICR mice. The splenocytes were treated with MOK extract (1.25, 2.5, 5, 10, and 20 mg/mL) for 30 min and then stimulated with Con A (200 ng/mL) for the indicated times. Cell viability of the splenocytes was measured using an MTT assay. The mRNA expression of Th1/Th2 cytokines (IFN-γ, IL-4, IL-10, and Foxp3) and antioxidant enzymes (HO-1 and MnSOD) was measured by RT-PCR.

Results: Addition of MOK extract at 2.5, 5, and 10 mg/mL in Con A–stimulated splenocytes significantly decreased the production of IFN-γ and significantly increased the expression of IL-4, IL-10, and Foxp3 mRNA. MOK extract also increased the mRNA expression of HO-1 and MnSOD in splenocytes.

Conclusion: MOK extract modulated the Th1/Th2 immune response via the regulation of cytokine levels in splenocytes and exerted an antioxidant effect via the upregulation of antioxidant proteins.

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I. Introduction

In order to efficiently overcome infections, immunomodulation boosts the immune system through the coordination of many immune cells. Splenocytes consist of different white blood cell types, such as T and B lymphocytes, dendritic cells, and macrophages, which have different immune functions. In the body, the spleen is an important immune organ that contains a relatively homogeneous fraction of B (60%) and T lymphocytes (40%). Thus, the immunomodulatory evaluation of splenocytes can provide an understanding of the effects of T and B cells.

Cytokines produced by CD4+ helper T (Th) lymphocytes are known to regulate the functions of the immune system, including antibody production and cellular immune response. Th cells represent a functionally heterogeneous population, composed of distinct subsets termed Th1 and Th2, which are defined by their cytokine secretion profiles. In general, cytokines produced by Th1 cells (e.g., IFN-γ and IL-2) promote the production of complement-fixing and opsonizing antibodies and macrophage activation. Cytokines produced by Th2 cells (e.g., IL-4, IL-5, IL-6, IL-10, and IL-13) have been reported to stimulate antibody production and promote mast cell and eosinophil granulocyte differentiation and activation. However, Th1 cytokines antagonize Th2 cell generation, and Th2 cytokines antagonize Th1 cell generation. Therefore, Th1 cells (IFN-γ) can exacerbate Th1-mediated autoimmune diseases, such as non-obese diabetic (NOD) diseases, rheumatoid arthritis, and Crohn’s disease, whereas Th2 cells (IL-4) can aggravate Th2-modulated disorders such as asthma.

Pharmacopuncture therapy is a method for the stimulation of acupoints through the injection of herbal medicines at the same acupoints. This method is frequently used to regulate immune balance in clinical settings. MOK is one of the pharmacopuncture medicines that is used to treat the meridian of fire in nature and clinical symptoms related to heart and thyroid diseases. MOK has been reported to exhibit anti-inflammatory and antioxidant effects in vitro. Among the constituents of MOK, Calculus Bovis from Bos taurus, Ursi Fel from Ursus arctos, and Hominis Placenta have been reported as herbs that may exert immunomodulatory effects. However, the scientific mechanism underlying the immunomodulatory effects of MOK is not fully known. Therefore, we investigated the immunomodulatory and antioxidant effects of MOK on Th1/Th2 imbalance and oxidative stress in primary Con A–stimulated mouse splenocytes.

II. Materials and Methods

1. Materials

1) Preparation of MOK extract
The MOK extract was manufactured at a Good Manufacturing Practice (GMP)-compliant facility at the Korea Immuno-Pharmacopuncture Association (Seoul, Korea). The quality of all the raw materials (Table 1) 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 h, mixed with ethanol 1:1 ratio, filtered through a two-layer mesh, and concentrated under vacuum pressure. Freeze-dried MOK was dissolved in 1X PBS to yield MOK extract at a concentration of 53.1 mg/mL.

2) Reagents
Concanavalin A (Con A), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), sulfanilamide, and N-1-naphthylethylenediamine dihydrochloride (NED) were purchased from Sigma–Aldrich (St Louis, MO, USA). RPMI–1640, fetal bovine serum (FBS), and penicillin–streptomycin solution were purchased from GenDEPOT.
(Barker, TX, USA). TRIzol reagent was obtained from Bio-Rad Laboratories (Philadelphia, PA, USA). M-MLV reverse transcriptase was obtained from Promega (Madison, WI, USA), Taq-based PCR enzyme was purchased from Toyobo (Osaka, Japan), and thioglycollate broth was purchased from Difco Laboratories (Detroit, MI, USA).

3) Animals

Five-week-old male ICR mice (weight: 17–21 g) were obtained from Orient Bio Inc. (Gyeonggi-do, Korea). The animals were housed under controlled environmental conditions (ambient temperature, 23 ± 1 °C; relative humidity, 50 ± 10%; 12-h light/dark cycle) and permitted free access to food and water.

2. Methods

1) Isolation of splenocytes

Spleens were rapidly harvested from mice, minced, and passed through a stainless steel mesh to obtain a single cell suspension. The cell pellet was harvested after the splenocytes were resuspended in 1× PBS and centrifuged at 5,000 rpm for 5 min. Erythrocytes were removed by using RBC lysis buffer (Sigma–Aldrich). Splenocytes (2 × 10^6 cells/mL) were cultured in 3 mL RPMI-1640 medium supplemented with 10% FBS, penicillin (100 units/mL), streptomycin (100 μg/mL), and β-mercaptoethanol (0.05 mM) at 37 °C in an atmosphere of 5% CO₂ for 24 h to facilitate cell adherence. Subsequently, the non-adherent cells were removed.

2) MTT assay

An MTT-based colorimetric assay was used to determine the concentration at which MOK extract was toxic to splenocytes. Briefly, splenocytes (5 × 10^4 cells/well) were seeded in 96-well plates and treated with MOK extract at different concentrations for 24 h at 37 °C in a 5% CO₂ incubator. Subsequently, 10 μL MT solution (5 mg/mL) was added to each well and incubated for 4 h. The resulting crystals were dissolved in 100 μL DMSO and the absorbance at 570 nm was measured using a microplate reader (GENios, TEKAN Instruments, Inc., Austria). Cell viability was calculated by using the following formula: (viable cells)% = (OD of MOK-treated sample/OD of untreated sample) × 100

3) Reverse Transcription (RT)–PCR assay

Splenocytes (1 × 10^6 cells/well) were seeded in 60-mm culture dishes, treated with MOK extract at 2.5, 5, and 10 mg/mL for 30 min, and then stimu-
lated in the presence or absence of Con A for 5 h at 37 °C in a 5% CO₂ incubator. The total RNA was isolated from each cell by using TRIzol reagent and cDNA was synthesized from total RNA by using a mixture of oligo–dT primer, 5X RT buffer (Promega Co., Madison, WI, USA), 0.5 mM dNTP, 3 mM MgCl₂, RNase inhibitor, and Improm–II reverse transcriptase (2U) at 25 °C for 5 min and 42 °C for 60 min. The reaction was terminated at 70 °C for 10 min. PCR was performed using specific primers for the target genes and PCR mixture [2 μL cDNA, 4 μM 5’ and 3’ primers, 10X buffer (10 mM Tris–HCl, pH 8.3), 50 mM KCl, 0.1% Triton X–100, 25 mM MgCl₂, 250 μM dNTPs, and 1 U Taq polymerase] under the following incubation conditions: 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 57–61 °C, and extension for 1 min; followed by a final extension step of 10 min. The following primer sequences were used: IL–4, 5’–AGA TGG ATG TGC CAA ACG TCC TCA–3’ (sense) and 5’–AAT ATG CGA AGC ACC TG TGG AAG GCC–3’ (anti–sense); IL–10, 5’–GGA CAA CAT ACT GCT AAC CGA C–3’ (sense) and 5’–TGG ATC ATT TCC GAT AAG GCT TG–3’ (anti–sense); Foxp3, 5’–GGC CCT TCT CCA GGA CAG A–3’ (sense) and 5’–GCT GAT CAT GGC TGG GTT GT–3’ (anti–sense); IFN–γ, 5’–TCA ACA ACC CAC AGG TCC AG–3’ (sense) and 5’–CTT CCT GAG GCT GGA TTC CG–3’ (anti–sense); MnSOD, 5’–GTG ACT TTG GGT CTT TTG AG–3’ (sense) and 5’–GCT GAT CAT TTC CAA GCC CAG A–3’ (anti–sense); HO–1, 5’–AAG ATT GAA GCC CAG AAA GCC CTG GAC–3’ (sense) and 5’–AAC TGT CGC CAC CAG AAA GCT GAG–3’ (anti–sense); and GAPDH, 5’–CTC GTG GAG TCT GCT GCT AG–3’ (sense) and 5’–AC TGT CGC CAC CAG AAA GCT GAG–3’ (anti–sense), which was used as a control for PCR. The band intensity was quantified by automated densitometric analysis (ChemiDoc MP Imaging System (BioRad Laboratories, CA, USA).

4) Statistical Analysis

GraphPad Prism (GraphPad Software, Inc., San Diego) was used for statistical analysis. Data were expressed as means ± SEM (standard error of mean) of three independent experiments and were analyzed for statistical significance by 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 in splenocytes

To investigate the cytotoxicity effects on splenocytes, the cells were treated with MOK extract between 1.25 mg/mL and 20 mg/mL and their viability was evaluated using an MTT assay. Cytotoxic effects were observed at 20 mg/mL MOK extract. However, no effects on the cell viability of splenocytes were observed after treatment with MOK extract in the range from 1.25–10 mg/mL (Fig. 1). Therefore, we used MOK extract at concentrations below 10 mg/mL for subsequent studies.

![Fig. 1. Effects of MOK extract on cell viability in mouse primary splenocytes](http://dx.doi.org/10.13045/acupunct.2017081)
2. Effects of MOK extract on the expression of IFN-γ in Con A-stimulated splenocytes

To understand the modulatory effect of MOK on the Th1/Th2 immune response, we evaluated the expression of the Th1 cytokine, IFN-γ, in Con A-stimulated splenocytes by RT-PCR. As shown in Fig. 2, the expression of IFN-γ mRNA was significantly increased after Con A stimulation (p < 0.001). Con A-induced IFN-γ expression was significantly decreased by the treatment with MOK extract at 2.5, 5, and 10 mg/mL (p < 0.05, p < 0.01, and p < 0.001, respectively). These data indicate that MOK modulated the Th1 response via the downregulation of IFN-γ expression in activated splenocytes.

3. Effects of MOK extract on the expression of IL-4, IL-10, and Foxp3 in Con A-stimulated primary splenocytes

To understand the modulatory effect of MOK on the Th1/Th2 immune response, we evaluated the expression of the Th2 cytokines, IL-4, IL-10, and Foxp3, in Con A-stimulated splenocytes by RT-PCR. The results indicated that the treatment with 2.5, 5, and 10 mg/mL MOK extract dose-dependently increased the expression of IL-4, IL-10, and Foxp3 in Con A-stimulated splenocytes (Fig. 3). In particular, a significant increase in IL-4, IL-10, and Foxp3 was observed at 10 mg/mL MOK extract. These results indicated that MOK modulated the Th2 response via the upregulation of IL-4, IL-10, and Foxp3 expression in activated splenocytes.

4. Effects of MOK extract on oxidative stress in primary splenocytes

To investigate the antioxidant effects of MOK extract in splenocytes, we analyzed the mRNA expression of antioxidant enzymes by using RT-PCR. The treatment of splenocytes with 10 mg/mL MOK extract significantly decreased the expression of the antioxidant enzymes, HO-1 and MnSOD mRNA (Fig. 4). These data indicated that MOK may exert an antioxidant effect in splenocytes through the upregulation of antioxidant enzymes.
Fig. 3. Effects of MOK extract on the expression of IL-4, IL-10, and Foxp3 mRNA in Con A–stimulated primary splenocytes

Splenocytes were treated with MOK extract at 2.5, 5, and 10 mg/mL for 30 min, and then stimulated in the presence or absence of Con A (200 ng/mL) for 5 h. (A) The expression of IL-4, IL-10, and Foxp3 mRNA was evaluated by RT-PCR assay, with GAPDH used as an internal control. (B) The histogram represents the means ± SD of three independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001 vs. cells only; †p < 0.05, ††p < 0.01, and †††p < 0.001 vs. Con A only.

Fig. 4. Effects of MOK extract on the expression of HO-1 and MnSOD mRNA in Con A–stimulated primary splenocytes

Splenocytes were treated with 10 mg/mL MOK extract for 30 min and then stimulated in the presence or absence of Con A (200 ng/mL) for 5 h. (A) The mRNA expression of HO-1 and MnSOD was determined by RT-PCR assay, with GAPDH used as an internal control. (B) The histogram represents the means ± SD of three independent experiments. *p < 0.05 vs. cells alone.
IV. Discussion

Acupuncture has been used to treat a variety of inflammatory conditions in the body. Growing evidence has indicated that acupuncture effectively improves various inflammatory diseases through immune regulation and anti-inflammatory effects. Pharmacopuncture is a new acupuncture treatment method in Traditional Korean Medicine (TKM). This method is often used in clinics because it can directly target tissues without involving the digestive system. MOK is one of the pharmacopuncture medicines that has been used to treat the meridian of fire in nature, clinical symptoms related to heart and thyroid diseases, and the Korean somatization disorder Hwa-Byung, which is a mental illness associated with the inability to control anger. MOK consists of ten herbs, including Hominis Placenta, Moschus, Ursi Fel, Bovis Calculus, Scutellariae radix, Phellodendri Cortex, Pulsatilla koreana, Sophorae subprostratae radix, Saussurea lappa, and Aquilaria agallocha. The main constituents are Moschus, Bovis Calculus (Bos taurus), Ursi Fel (Ursus arctos), and Hominis Placenta. The immunomodulatory function of these herbs has been reported in modern pharmacological research along with antioxidant effects. However, comparisons of the biological activity of MOK with that of single medicines have been rarely reported. We have previously reported the anti-inflammatory and antioxidant effects of MOK extract in activated peritoneal macrophages. In the present study, we investigated the effects of MOK extract on the Th1/Th2 immune imbalance and oxidative stress in primary splenocytes isolated from mouse spleen. The treatment of Con A-stimulated splenocytes with MOK extract decreased the expression of the Th1 cytokine, IFN-γ, and increased the expression of the Th2 cytokines, IL-4, IL-10, and Foxp3. These changes indicated that MOK extract was able to control immune imbalance via the regulation of the Th1/Th2 response in splenocytes.
 immunopathology for all types of infections\textsuperscript{30,31}. In the present study, treatment with MOK significantly increased the expression of Foxp3 mRNA in Con A-stimulated splenocytes. This result suggested that MOK extract may control the autoimmune response through the activation of Treg cells. Autoimmune diseases may be caused by an imbalance of Th1/Th2 cytokines and regulatory cytokines. In further studies, we will investigate the effects of MOK extract on Treg cell number and function in thyroid autoimmune diseases.

Oxidative stress induced by ROS overproduction, which results in damaged cellular lipids, proteins, and DNA, is thought to be implicated in a variety of diseases or pathological conditions including cancers, cardiovascular disease, acute inflammatory problems, complications of diabetes mellitus, chronic inflammatory diseases, central nervous system disorders, neurodegenerative disorders, and age-related disorders\textsuperscript{32,33}. The endogenous components of the antioxidant system include enzymes such as SOD, glutathione peroxidase (GSPx), and HO–1\textsuperscript{34,35}. We previously reported that the antioxidant effects of MOK extract in LPS–stimulated mouse peritoneal macrophages occurred via an increase in the expressions of MnSOD and HO–1\textsuperscript{7}. In the present study, the treatment of splenocytes with MOK increased the mRNA expression of MnSOD and HO–1. These results indicated the antioxidant potential of MOK extract.

V. Conclusions

In conclusion, MOK extract inhibited the mRNA expression of the Th1 cytokine IFN–γ, induced the expression of Th2 cytokines (IL–4, IL–10, and Foxp3), and also induced the mRNA expression of the antioxidant enzymes SOD and HO–1 in Con A–stimulated mouse splenocytes. This suggested that MOK extract exerted immunomodulatory and antioxidant effects in splenocytes through the regulation of Th1/Th2 cytokines and antioxidant enzymes.

VI. References

33. Sareen SG, Jack LS, James LG. The antioxidant
