한방안이비인후피부과학회지 제19권 제2호(2006년 8월)
The Journal of Korean Oriental Medical Ophthalmology & Otolaryngology & Dermatology 2006;19(2) : 118-129

The Anti-inflammatory Effects of Seunggaltang(升葛湯) in the BV2 cells

Su-Kyung Jun · Hwa-jung Yoon · Woo-Shin Ko* · Hoon Kim**
* Dept. of The Oriental Medical Ophthalmology & Otolaryngology & Dermatology, College of Oriental Medicine, Dong Eui University
** Dept. of Medical History Collegy of Korean Medicine, Dongeui University

升葛湯의 抗炎症효과에 대한 연구

전수경 · 윤화정 · 고우신 · 김 훈

두드러기는 가려움으로 인해 발생되는 피부상의 상해질환으로써 나이에 관계없이 높은 발병률을 가지고 있다. 한방에서는 만성두드러기의 원인 중 하나로 脾胃의 濕熱을 말한다. 升葛湯은 補脾益氣의 효능을 가지고 있어 脾胃의 濕熱을 없애는데 사용되어 왔다.

최근 연구에 의하면 升葛湯은 재발성 홍반성 두드러기를 효과적으로 억제하는 효능이 있다고 밝혀졌으나 升葛湯의 항염증효과에 대한 메커니즘은 명백하지 않은바 이에 우리는 BV2세포에 있어서 NO농도, iNOS의 발현정도, PGE_2의 생산, 세포생존도, COX-2, IL-1β, IL-12, TNF-α의 발현을 조사하였다.

그 결과 升葛湯은 NO, PGE_2의 생산을 현저하게 억제하며 iNOS, COX-2, IL-1β, IL-12, TNF-α 역시 감소하였고 세포생존도에는 영향을 주지 않는 것으로 나타났다. 以上을 살펴볼 때 升葛湯은 항염증 효과를 가지고 있음을 알 수 있다.

Key words : Urticaria, Seunggaltang, iNOS, COX-2, PGE_2, IL-1β, IL-12, TNF-α

Ⅰ. Introduction

Chronic urticaria (CU) is a common problem and 15.20% of the population will experience one or more episodes of urticaria in a lifetime. More than half of the patients will have hives for longer than 1 year with 20% of these continuing to have the disease for up to 20 years or longer. CU has been shown to be a cause of serious personal, economic, social and occupational disability comparable with that associated with severe coronary artery

교신저자 : 윤화정, 부산광역시 부산진구 양정2동 산 45-1 동의의료원, Tel : 051-850-8658, E-mail : yhj1226@deu.ac.kr
※ 본 연구는 보건복지부 한방치료기술연구개발사업의 지원에 의하여 이루어진 것임 (B050008).
• 접수 2006/06/12 • 수정 2006/07/18 • 채택 2006/08/04

disease[1].

It has been reported that Spleen-Stomach system has power to control total human body and spirit in Traditional Korean Medicine. In other words, it is that treats diseases through to supply energy to deficiency other organism[2]. And so it is regarded totally as to improve weakness and to remove foreign invader, including inflammatory factors and viral infection, and so this herbal drug may affect to the key parameters of inflammation as production of nitric oxide (NO), cyclooxygenase (COX)-2, prostaglandin E_2, and proinflammatory cytokines.

Seunggaltang (SKT) had been made to as purpose to remove the damp-heat (濕熱) of the Spleen-Stomach system from Seungmagalgeuntang in the Dongeui Oriental Hospital prescription. Recently, Yoon etc reported Seunggaltang effectively suppresses recurrent, pruirtus and erythematous urticaria[3,4]. However, anti-inflammatory effects by SKT have not been clarified on the microglial cells. The aims of the present study demonstrates the effects of SKT on the expression level of iNOS mRNA, COX-2 mRNA, PGE_2, and proinflammatory cytokines from LPS-stimulated mouse BV2 microglial cells.

II. Materials and Methods

2.1. Preparation of SKT aqueous extracts

Dried Oriental medicinal herbs used for preparation of SKT were purchased from Oriental Medicine Hospital, Dongeui University (Busan, Korea). SKT (total 164g), a one day dose for human adults were boiled with 1200 ml distilled water at 100 ℃, and the whole mixture is decocted until the volume is reduced by half. The extract water (600 ml) was filtered through 0.45 μm filter and the filtrate was freeze-dried (yield, 7g) and kept at 4 ℃. The dried filtrate was dissolved in phosphate buffered saline (PBS) and filtered through 0.22 μm filter before use.

Table 1. Prescription of SKT

Herbs	Scientific name	Dose
金銀花	*Lonicerae Flos*	12g
浮萍草	*pirodelae Herba*	10g
葛　根	*Puerariae Radix*	8g
山査肉	*Crataegi Fructus*	8g
蘿蔔子	*Raphani Semen*	6g
升　麻	*Cimicifugae Rhizoma*	4g
白芍藥	*Paeoniae Radix*	4g
黃　芩	*Scutellariae Radix*	4g
連　翹	*Forsythiae Fructus*	4g
陳　皮	*Aurantii nobilis Pericarpium*	4g
厚　朴	*Magnoliae officinalis Cortex*	4g
枳　實	*Ponciri Fructus*	4g
竹　茹	*Bambusae silicea Concretio*	4g
甘　草	*Glycyrrhizae Radix*	4g
薄　荷	*Menthae Folium*	2g
	Total mount	82g

2.2. Reagents

LPS (phenol extracted *Salmonella enteritidis*), Tween-20, bovine serum albumin (BSA), phosphatase-conjugated goat anti-rabbit IgG, and *p*-nitrophenyl phosphate, 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). 24-well, 96-well tissue culture plates and 60 mm culture dishes were purchased from Nunc, Inc. (North Aurora Road, IL). DMEM containing

L-arginine (200 mg/L), FBS, and other tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD). All reagents and media for tissue culture experiments were tested for their LPS content with use of a colorimetric *Limulus* amoebocyte lysate assay (detection limit, 10 pg/ml; Whittaker Bioproducts, Walkersville, MD).

2.3. Cells culture

The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin, and were maintained in a humidified incubator with 5% CO_2 atmosphere. Cells were plated at a density of 2×10^5 cells/well in a 60mm dish, and allowed to attach for 2 h. In all experiments, cells were treated with SKT for 1 h in the presence of the indicated concentrations of SKT before the addition of LPS (1 μg/mL) in serum-free DMEM.

2.4. Measurement of nitrite concentration

The production level of NO in cell cultures was measured by a microplate assay method, as described previously[5]. After cells were stimulated in 24 well for 24 h, 100 μl each cultured medium was mixed with the equal volume of the Griess reagent (1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10min. NO concentration was determined by measuring the absorbance at 540 nm with a Vmax 96-well microplate spectrophotometer. Nitrite was determined by using sodium nitrite as standard. The level of nitrite reflelcts NO synthesis.

2.5. Cytotoxicity assay

The cytyotoxicity of SKT was assessed using the MTT assay[6] in the remaining cells after Griess reaction. 0.5 mg/ml of MTT solution [3-(4,5-dimethylthiaozle-2-yl)-2,5-diphenyl-tetrazolium bromide] was added to each well. After incubation for 2 h at 37 °C and 5% CO_2, the supernatant was removed and the formed formazan crystals in viable cells were measured at a wave-length 540 nm with spectrophotometric plate reader. The percentage of cytotoxicity was calculated against untreated cells. All experimental dilutions of SKT were performed in triplicate wells.

2.6. RNA isolation from cells and RT-PCR

After the treatment with LPS in the presence or absence of SKT, 2×10^5 cells were washed and harvested. Total RNA was isolated as per the manufacture's instructions. Briefly, cells were lysed by the addition of Trizol and the cell lysate was passed through the pipette several times. 0.2ml of chloroform was added per 1ml of Trizol reagent. The tubes were shaken vigorously and incubated at room temperature for 2-3 min. The samples were centrifuged at 14,000g for 20 min. The aqueous phase was transferred to a fresh tube and RNA was precipitated by the addition of 0.5ml isopropanol. The RNA pellet was air-dried and resuspended in nuclease-free water. The concentration of RNA was estimated spectrophotometrically. Three micrograms RNA were reverse-transcribed using MMLV reverse transcriptase (Promega). Single stranded cDNA

was amplified by PCR with primers (Table 2). PCR amplifications were done in a 20 ㎕ PCR PreMix (Bioneer Co. Daejon, Korea) containing 10 mM Tris-HCl, 40 mM KCl, 1.5 mM $MgCl_2$, 250 ㎛M dNTP, 1 unit of *Taq* polymerase. Amplifications were carried out in a PCR machine (ASTEC PC802, Japan) using an initial denaturation at 94 ℃ for 5 min followed by 30 cycles of denaturation for 30 sec at 94 ℃, annealing for 30 sec at 52 ℃ (56 ℃ for GAPDH) and extension for 1 min at 72 ℃. This was concluded with a final extension for 5 min at 72 ℃. Amplicons were separated in 1 % agarose gels in 1× TBE buffer at 100 V for 30 min, stained with ethidium bromide and visualized under UV light.

Table 2. Primers used in this study

primers		sequence (5' → 3')
iNOS	sense	GAC-AAG-CTG-CAT-GTG-ACA-TC
	antisense	GCT-GGT-AGG-TTC-CTG-TTG-TT
Cox-2	sense	TTG-AAG-ACC-AGG-AGT-ACA-GC
	antisense	GGT-ACG-GTT-CCA-TGA-CAT-CG
IL-1β	sense	TGA-AGG-GCT-GCT-TCC-AAA-CC
	antisense	TGT-CCA-TTG-AGG-TGG-AGA-GC
IL-12	sense	ACC-TCA-GTT-TGG-CCA-GGG-TC
	antisense	GTC-ACG-ACG-CGG-GTG-GTG-AA
TNF-α	sense	GCG-ACG-TGG-AAC-TGG-CAG-AA
	antisense	TCC-ATG-CCG-TTG-GCC-AGG-AG
GADPH	sense	GGT-GAA-GGT-CGG-TGT-GAA-CG
	antisense	GGT-AGG-AAC-ACG-GAA-GGC-CA

Statistical analysis

Data is presented as the mean±SD of at least three separate experiments. Comparisons between two groups were analyzed using Student's *t*-test. P values less than 0.05 considered to be statistically significant.

Ⅲ. Results

3.1. Inhibitory effect of SKT in NO production from LPS-induced BV2 microglial cells

Nitric oxide (NO) is a short-lived free radical that mediates many biological functions[7]. However, high level of NO production might cause host cell death and inflammatory tissue damage[8-11].

Thus, the production of NO represents a potential double-edged sword. We investigate the effects of SKT on NO production in LPS-stimulated BV2 microglial cells. Cells were pre-incubated in 24 well tissue culture plates (2×10^5 cells/well) with 0.25, 0.5, 1, and 2 mg/ml SKT for 1 h and stimulated with 1 μg/ml of LPS for 24 h. Non-treat cells and SKT-treated cells without LPS produced 4.5±2.3 and 6.4±3.2 μM of NO. LPS-stimulated

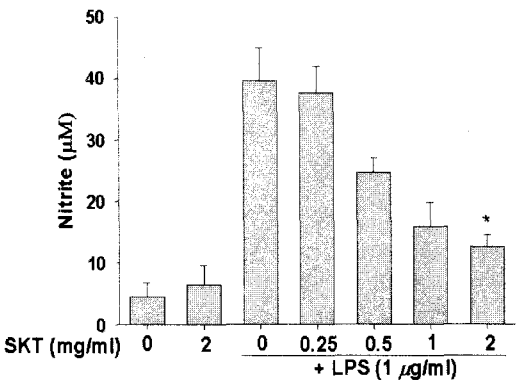

Fig. 1. Eeffects of SKT on the LPS-induced NO production

Cells were treated with the indicated concentration (0.25, 0.5, 1, 2 mg/ml) of SKT for 1 h. Then, the cells were stimulated with LPS(1 μg/ml). After cells were incubated for 24 h, NO release was measured as a form of nitrite by the Griess method. NO(nitrite) released into the medium is presented as the mean±S.E. of three independent experiments duplicate in each run.

* $P<0.05$, significantly different from the LPS.

cells without SKT (control cells) produced 39.5± 5.4 μM. When the cells were treated with 0.25, 0.5, 1, and 2 mg/ml SKT, the cells produced 37.6±4.3, 24.7±2.4, 15.8±3.9, 12.6±1.9 μM NO, respectively. SKT concentration-dependently inhibited the LPS-induced NO release into culture supernatant (Fig. 1).

3.2. SKT inhibits iNOS mRNA expression on the LPS-stimulated BV2 microglial cells

Since NO is a free radical generated through the conversion of L-arginine to citrulline, catalysed by NO synthase (NOS), we investigated whether the inhibition of NO production by SKT was the result of the inhibition of iNOS mRNA. The inhibitory effects of the indicated concentration (0.25, 0.5, 1, 2 mg/ml) of SKT in iNOS protein expression induced by LPS (1 μg/ml). The cells were incubated with 0.25, 0.5, 1, and 2 mg/ml SKT for 1 h, respectively. Then, BV2 microglial cells were stimulated with LPS (1 μg/ml) for 6 h. In this study, the mRNA level of iNOS in the control cells was set as 1.00. Non-treat cells and SKT-treated cells without LPS expressed 0.00 and 0.30 density in comparison with the control. When the cells were treated with 0.25, 0.5, 1, and 2 mg/ml SKT, the cells expressed 0.90, 0.98, 0.75, and 0.39 densities in comparison with the control, respectively. As shown in Fig. 2, SKT inhibited iNOS protein expression at 1, 2 mg/ml SKT blocked iNOS induction by LPS. But level of iNOS expression at 0.25, 0.5 mg/ml are fewer than 1, 2 mg/ml.

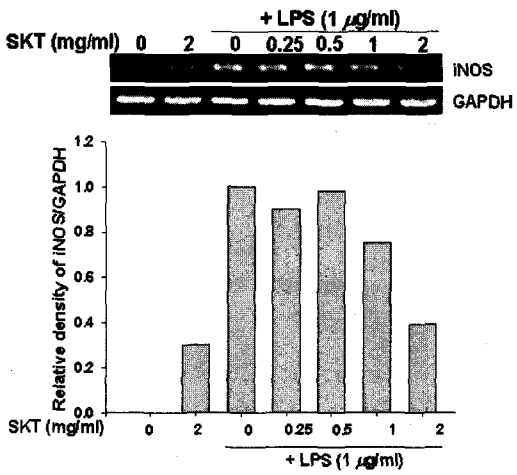

Fig. 2. Effects of SKT on the LPS-induced iNOS mRNA expression

Cells were treated with the indicated concentration (0.25, 0.5, 1, 2 mg/ml) of SKT for 1 h. Then, the cells were stimulated with LPS(1 μg/ml). After cells were incubated for 6 h, total RNA was prepared. And iNOS mRNA was analyzed by RT-PCR. GAPDH was used as control genes.

3.3. SKT inhibits PGE_2 synthesis from LPS-induced BV2 microglial cells

Prostaglandins(PGs) are well-known proinflammatory mediators in the pathogenesis of inflammation[12]. Therefore, we investigated the effect of SKT on LPS-induced PGE_2 production. The cells were pretreated with 0.25, 0.5, 1, or 2 mg/ml SKT for 1 h and stimulated with 1 μg/ml LPS for 24 h. Non-treat cells and SKT-treated cells without LPS produced 0.5±0.1 and 0.3±0.1 μM PGE_2, respectively. Control cells produced 3.0±0.3 μM. When the cells were treated with 0.25, 0.5, 1, 2 mg/ml SKT, the cells produced 3.0±0.2, 2.5±0.5, 2.0±0.1, and 1.1±0.2 μM PGE_2, respectively. SKT suppressed PGE_2 release into culture supernatant in a dose-dependent manner (Fig. 4). PGE_2 production was significantly decreased in 2 mg/ml concentration of SKT.

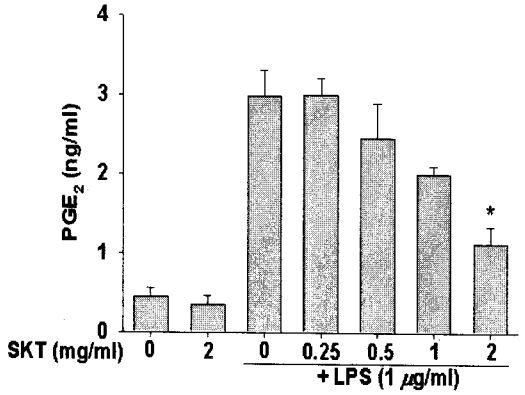

Fig. 3. Inhibition effect of SKT on the PGE2 production on the LPS-stimulated BV2 microglial cells

Cells were treated with the indicated concentration (0.25, 0.5, 1, 2 mg/ml) of SKT for 1 h. Then, the cells were stimulated with LPS(1 μg/ml). After cells were incubated for 24 h. The production of PGE_2 concentration was determined by ELISA. PGE_2 concentration into the medium is presented as the mean±S.E. of three independent experiments duplicate in each run.

* $P<0.05$, significantly different from the LPS.

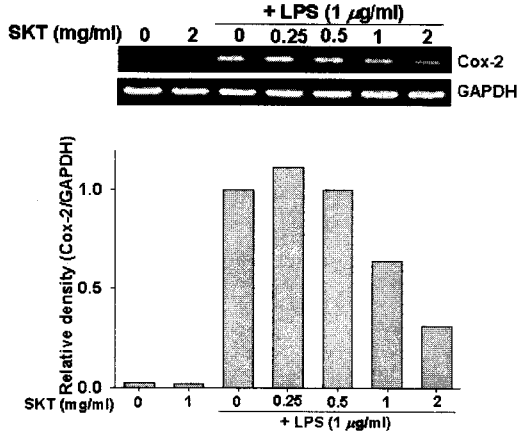

Fig. 4. Inhibitory effects of SKT on the LPS-induced COX-2 mRNA expression

Cells were treated with the indicated concentration (0.25, 0.5, 1, 2 mg/ml) of SKT for 1 h. Then, the cells were stimulated with LPS(1 μg/ml). After cells were incubated for 6 h, total RNA was prepared. And COX-2 mRNA was analyzed by RT-PCR. GAPDH was used as control genes.

3.4. Inhibitory effect of SKT on expression of COX-2 mRNA from LPS-induced BV2 microglial cells

PGs are synthesized from arachidonic acid by a reaction catalyzed by cyclooxygenase. Two isoforms of this enzyme have been identified[13]. COX-1 is expressed constitutively in almost all tissues[14], and COX-2 is an inducible enzyme that is expressed in response to inflammatory cytokines[15]. According to the results (Fig. 3), we investigated whether it also inhibits expression of COX-2 mRNA. The cells were pretreated with 0.25, 0.5, 1, and 2 mg/ml SKT for 1h, respectively, after incubated for 6 h in the presence of 1 μg/ml LPS. The mRNA level of COX-2 in the control cells was set as 1.00. Non-treat cells and SKT-treated cells without LPS expressed 0.02 and 0.02 density in comparison with the control. When the cells were treated with 0.25, 0.5, 1, and 2 mg/ml SKT, the cells expressed 1.11, 1.00, 0.64, and 0.31 densities in comparison with the control, respectively. This results indicated that LPS-induced expression of the COX-2 mRNA was decreased significantly by SKT (Fig. 4).

3.5. Affect of SKT on the Cytotoxicity by MTT assays

The cytotoxicity effect of SKT on BV2 microglial cells was evaluated in the presence or absence of LPS by MTT assay. As shown in Fig. 5, SKT at concentrations from 0.25 mg/ml to 2 mg/ml had no effect on cell survival. These results suggest SKT inhibits LPS-induced NO and PGE_2 production in BV2 microglial cells without effect on the cell viabiliy in each condition.

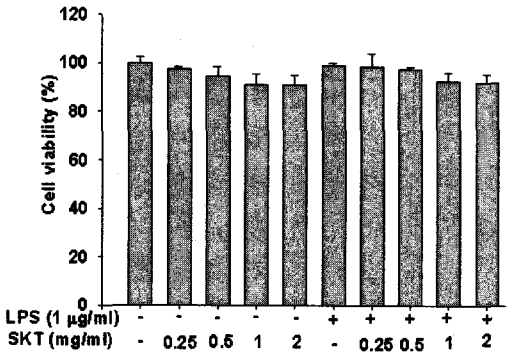

Fig. 5. Cell viability effects of SKT on the LPS-stimulated BV2 microglial cells

Cells were treated with the indicated concentration (0.25, 0.5, 1, 2 mg/ml) of SKT for 1 h. Then, the cells were stimulated with LPS(1 μg/ml). After cells were incubated for 24 h. Cell viability was evaluated in both floating and attaching cells using a colorimetric assay based on the MTT assay.

3.6. SKT inhibits IL-1β, IL-12, TNF-α mRNA expression on the LPS-induced BV2 microglial cells

To elucidate the effect of SKT on the LPS-induced expression of proinflammatory cytokines such as IL-12, TNF-α, and IL-1β mRNA, the cells were treated with 0.5, 1, and 2 mg/ml SKT for 1 h, respectively, after incubated for 6 h in the presence of 1 μg/ml LPS. In this study, the mRNA level of IL-12, TNF-α, and IL-1β in the control cells was set as 1.00. IL-1β mRNA level, non-treat cells and SKT-treated cells without LPS expressed 0.02 and 0.02 density in comparison with the control. When the cells were treated with 0.5, 1, and 2 mg/ml SKT, the cells expressed 0.45, 0.37, and 0.14 densities, respectively (Fig. 6). In the IL-12 mRNA expression level, non-treat cells and SKT-treated cells without LPS expressed 0.03 and 0.00 density. When the cells were treated with 0.5, 1, and 2 mg/ml SKT, the cells expressed 0.50,

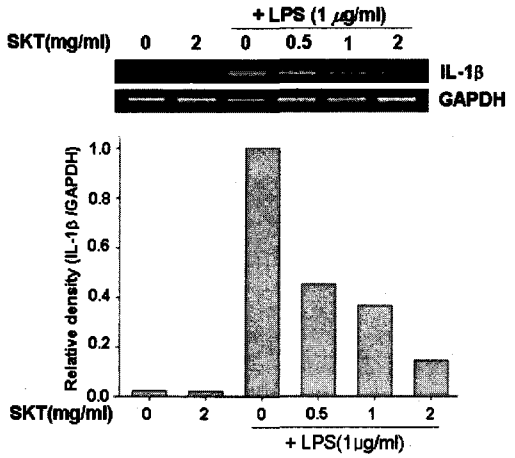

Fig. 6. Inhibitory effects of SKT on IL-1β mRNA level in the LPS-stimulated BV2 microglial cells

Cells were treated with the indicated concentration (0.5, 1, 2 mg/ml) of SKT for 1 h. Then, the cells were stimulated with LPS(1 μg/ml). After cells were incubated for 6 h, total RNA was prepared. And IL-1β mRNA was analyzed by RT-PCR. GAPDH was used as control genes.

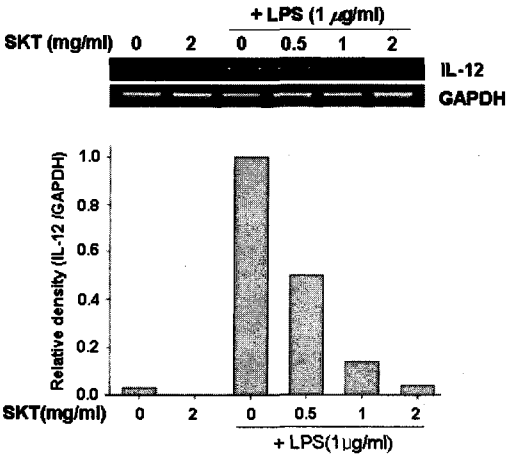

Fig. 7. Inhibitory effects of SKT on IL-12 mRNA level in the LPS-stimulated BV2 microglial cells

Cells were treated with the indicated concentration (0.5, 1, 2 mg/ml) of SKT for 1 h. Then, the cells were stimulated with LPS(1 μg/ml). After cells were incubated for 6 h, total RNA was prepared. And IL-12 mRNA was analyzed by RT-PCR. GAPDH was used as control genes

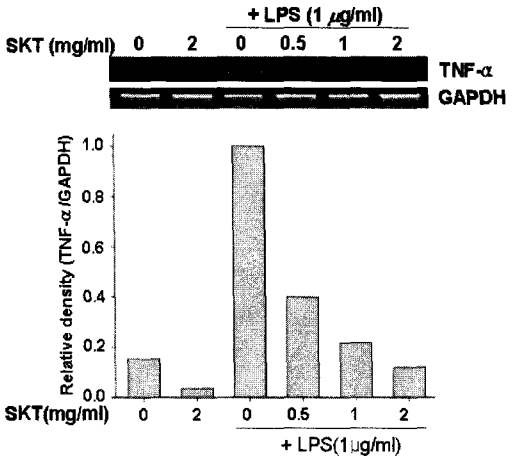

Fig. 8. Inhibitory effects of SKT on TNF-α mRNA level in the LPS-stimulated BV2 microglial cells

Cells were treated with the indicated concentration (0.5, 1, 2 mg/ml) of SKT for 1 h. Then, the cells were stimulated with LPS(1 μg/ml). After cells were incubated for 6 h, total RNA was prepared. And TNF-α was analyzed by RT-PCR. GAPDH was used as control genes.

0.14, and 0.04 densities (Fig. 7). And in the TNF-α mRNA level , non-treat cells and SKT-treated cells without LPS expressed 0.15 and 0.04 density. When the cells were treated with 0.5, 1, and 2 mg/ml SKT, the cells expressed 0.40, 0.22, and 0.12 densities (Fig. 8).

Ⅳ. Discussion

Urticaria is a cutaneous lesion characterised by the appearance of pruritic wheal and flare reactions, commonly called hives. A similar process may also take place at mucosal surfaces. Urticaria can occur at any age, with the highest incidence in the third decade of life. Chronic urticaria (CU) is defined as the repeated occurrence of wheals at least three times a week lasting less than 24 h over a period of more than 6 weeks[16,17]. One of the chronic urticaria's cause in the Korean Traditional Medicine is due to damp-heat (濕熱) of the Spleen-Stomach[3]. SKT has been used to remove the damp-heat of the Spleen-Stomach through to tonify the spleen and replenish Ki (補脾益氣) effects.

SKT had been made to as purpose to remove the damp-heat of the Spleen-Stomach from Seungmagalgeuntang prescription in the Dongeui Oriental Hospital. Recently, Yoon etc reported SKT effectively suppresses recurrent, pruritus and erythematous urticaria[3,4]. However, the regulation mechanism of anti-inflammatory effects by SKT has not been clarified. Therefore, in this study, we investigated that SKT induces anti-inflammatory effects in BV2 microglial cells.

Ingredients of SKT are as follow; *Lonicerae Flos* (金銀花) and *Forsythiae Fructus* (連翹) exerts effects of clear away heat and toxic material (淸熱解毒), *Spirodelae Herba* (浮萍草) has not only expel wind, promote diuresis (祛風行水) but also clear away of toxic material via dispersing actions of sweet (發汗解毒). *Puerariae Radix* (葛根) has dispel pathogenic factors from the superficial muscle and reduce heat (解肌退熱) and promote eruption (透疹). *Crataegi Fructus* (山査肉) promote digestion and remove food stagnancy (消食導滯). *Cimicifugae Rhizoma* (升麻) dispel pathogenic factors from the exterior of the body (解表) and clear away heat and toxic material (淸熱解毒). *Raphani Semen* (蘿蔔子) remove stagnation of Ki and resolve phlegm (消積化痰). *Paoniae radix alba* (白芍藥) tonify the blood (補血). *Scutellariae Radix* (黃芩) dliminate heat and dampness (淸熱燥濕), purge of fire and detoxicate (瀉火解毒). *Aurantii nobilis*

Pericaroium (陳皮) regulate the flow of Ki and invigorate the spleen (理氣健脾), also have eliminate dampness and reslove eruption (燥濕化痰). *Magnoliae officinalis Cortex* (厚朴) eliminate dampness and relieve distension(燥濕除滿), promote the flow of Ki to alleviate stagnancy in the spleen and stomach (行氣寬中). *Panciri Fructus* (枳實) promote the flow of Ki to relieve distension (行氣消脹). *Bambusae silicea Concretio* (竹茹) clear away heat (淸熱), resolve phlegm (化痰) and arrest vomitting (止嘔). *Glycyrhizae radix* (甘草) regulates whole herbs. *Menthae Folium* (薄荷) dispel wind and heat (疏散風熱)[18].

Lipopolysaccharide (LPS) is a component of the cell wall of Gram-negative bacteria[19]. Inflammatory stimuli, such as LPS, induce cytokines in the process of macrophage activation, which mediates tissue responses in different phases of inflammation in a sequential and concerted manner[20]. Exposure to LPS causes inflammatory liver damage and septic shock due to production of high levels of these cytokines[22]. Thus, inhibition of cytokine production or function serves as a key mechanism in the control of inflammation[21].

Nitric oxide (NO) is a short-lived free radical that mediates many biological functions[7]. However, high level of NO production might cause host cell death and inflammatory tissue damage[8-11]. This strong inflammatory response to foreign cells could also cause further damage for the neighboring cells and tissues of the host[23]. NO is produced from conversion of L-arginine to citrulline *in vivo* by three distinct isoforms of NO synthase (NOS): neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). NOSs present in the vascular endothelium (eNOS) and in central and peripherial neurons (nNOS) are constitutive (cNOS). iNOS isoform is an important enzyme involved in the regulation of inflammation[24]. Therefore, the reduction of these harmful effects is seemed to be important in inflammation therapy.

In the shown Fig. 1, SKT significantly inhibited LPS-induced NO production in BV-2 microglia without appreciable cytotoxic effects (Fig. 3). These results suggest that SKT could do potent anti-inflammatory action via inhibition of NO release. But, it is not known whether the reduction in nitrite accumulation by SKT is a result of inhibition of iNOS expression. Therefore, it was investigated whether SKT could affect iNOS mRNA and protein levels. The expression level of iNOS mRNA was gradually decreased by treatment of SKT in a dose dependent manner (Fig. 2). This result implicates that a significant decrease in NO release by SKT is linked to the expression level of iNOS gene.

PGE_2, a major metabolite of the COX-2 pathway and immunoregulatory processes. PGE_2 has been implicated in the pathogenesis of acute and chronic inflammatory disease states[25]. We investigated that SKT suppressed PGE_2 production in the BV2 microglial cells (Fig. 3). But, it is not known whether the reduction in PGE_2 production by SKT is a result of inhibition of COX-2 expression or inhibition of its enzymatic activity. Therefore, it was investigated whether SKT could affect COX-2 mRNA levels. To determine whether the decreased PGE_2 production is correlated with COX-2 expression, COX-2 mRNA and protein level was analyzed by RT-PCR. The expression of COX-2 mRNA was gradually decreased by

treatment of SKT in a dose dependent manner (Fig. 4). This result implicates that a significant decrease in PGE_2 release by SKT is linked to the expression level of COX-2 gene.

Pro-inflammatory cytokines such as TNF-α and IL-1β are strong stimulators and/or co-stimulators for iNOS gene expression in certain cell types[26,27]. TNF-α has been found to act synergistically on NO production and blocking of TNF-α by a specific antibody to indirectly decreases NO synthesis, impairing cell's defense against parasites[28,29]. Therefore SKT may inhibit the production of NO by suppressing TNF-α secretion.

IL-12 is a heterodimeric cytokine which is produced by activated antigen-presenting cells (APC), such as dendritic cells (DC), monocytes/ macrophages and microglia, in response to bacterial products and immune signals[30].

In conclusion, the present study has demonstrated that SKT is involved in the LPS-stimulated NO, COX-2, and proinflammatory cytokines production. As the effect of SKT is mediated via inhibition of iNOS, the oriental herbal prescription could perhaps be used in pathological processes in which induction of NO is involved. Also septic shock, arthritis and trauma seem to be promising targets, as the production of TNF-α is also inhibited by SKT.

This results improve that SKT could be used as an potent anti-inflammatory herbal prescription.

V. Conclusion

To evaluate the effect of SKT on the LPS-induced BV2 microglial cells, we observed NO concentration, expression level of iNOS mRNA, PGE_2 production, expression COX-2 mRNA, cell viability, IL-1β, IL-12, TNF-α gene expression. The results were as followed.

1. SKT suppressed significantly NO production.
2. Expression level of iNOS mRNA was reduced by SKT.
3. SKT suppressed significantly PGE_2 production.
4. Expression level of COX-2 mRNA was reduced by SKT.
5. Cell viability was not affected by SKT.
6. Expression level of IL-1β mRNA was suppressed by SKT
7. SKT suppressed expression of IL-12 mRNA
8. Expression level of TNF-α mRNA was decreased by SKT

These results suggest that SKT has anti-inflammatory effects on the BV2 microglial cells.

Acknowledgement

This study was supported by a grant of the Oriental Medicine R&D Project, Ministry of Health & Welfare, Republic of korea(BK050008).

References

1. O' Donnell, B.F., Lawlor, F., Simpson, J., Morgan, M., Greaves, M.W., 1997. The impact of chronic urticaria on the quality of life. Br. J. Dermatol. 136, 197-201.
2. The pathology society of Oriental medicine

university of the whole aspect. Oriental medicine pathology. Haneuimunhwasa. 2001: 308.

3. Yoon, J.W., Yoon, S.W., Yoon, H.J., Ko, W.S., 2003. The clinical study of urticaria. J. of Korean Oriental Med. Opthalmo. & Otolaryngo. & Derm. 16(2), 152-176.
4. Yoon, S.W., Kim, M.H., Yoon, H.J., Ko, W.S., 2004. A clinical study about the effect of sungmagalguntanggamibang on urticaria. J. of Korean Oriental Med. Opthalmo. & Otolaryngo. & Derm. 17(3), 61-79.
5. Schmidt, H.H.H.W., Kelm, M., 1996. Determination of nitrite and nitrate by the Griess reaction. In: Methods in Nitric Oxide research, John Wiley, Sons Ltd., 491-497.
6. Skehan, P., 1998. Assays of cell growth and cytotoxicity. In: Studzinski, G. P. (Eds.), Cell growth and apoptosis. Oxford University press, New York, 180.
7. Moncada, S., Palmer, R.M., Higgs, E.A., 1991. Nitric oxide: Physiology, pathophysiology, and pharmacology. Pharmacol. Rev. 43, 109-142.
8. Ishihare, T., Okura, T., Kohno, K., Tanimoto, T., Ikegami, H., Kurimoto, M., 2000. Polygonum tinctorium extract suppresses nitric oxide production by activated macrophages through inhibiting inducible nitric oxide synthase expression. Journal of Ethnopharmacology 72, 141-150.
9. Kim, N.Y., Kang, T.H., Song, E.K., Pae, H.O., Chung, H.T., Kim, Y.C., 2000. Inhibitory effects of butanol fraction of the aqueous extract of Forsythia koreana on the nitric oxide production by murine macrophage-like RAW 264.7 cells. Journal of Ethnopharmacology 73, 323-327.
10. Seo, W.G., Pae, H.O., Oh, G.S., Chai, K.Y., Kwon, T.O., Yun, Y.G., Kim, N.Y., Chung, H.T., 2001a. Inhibitory effects of methanol extract of Cyperus rotundus rhizomes on nitric oxide and superoxide productions by murine macrophage cell line, RAW 264.7 cells. Journal of Ethnopharmacology 76, 59-64.
11. Seo, W.G., Pae, H.O., Oh, G.S., Chai, K.Y., Yun, Y.G., Kwon, T.O., Chung, H.T., 2001b. Inhibitory effect of ethyl acetate fraction from Cudrania tricuspidata on the expression of nitric oxide synthase gene in RAW 264.7 macrophages stimulated with interferon-and lipopolysaccharide. General pharmacology 35, 21-28.
12. Vane, J. R., Mitchell, J. A., Appleton, I., Tomlinson, A., Bishop-Bailey, D., Croxtall, J., Willoughby, D. A., 1994. Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation. Proc. Natl. Acad. Sci. U.S.A. 91, 2046-2050.
13. Adler, K.B., Fischer,B.M., Wright, D.T., et al.: 1994. Interactions between respiratory epithelial cells and cytokines: relationships to lung inflammation. Ann. N. Y. Acad. Sci. 725, 128-145.
14. Raz, A., Wyche, A., Siegel, N., et al.: 1988. Regulation of fibroblast cyclooxygenase synthesis by interleukin-1. J. Biol. Chem. 263, 3022-3028.
15. Dubois, R.N., Abramson,S.B., Crofford,L., et al.: 1998. Cyclooxygenase in biology and disease. FASEB J. 12, 1063-1073.
16. Champion, R.H., 1990. A practical approach to the urticarial syndromes. a dermatologist' s

view. Clin. Exp. Allergy 20, 221-224.

17. Greaves, M.W., 1995. Chronic urticaria. N. Engl. J. Med. 332, 1767.1772.
18. Shin, M.K., An, D.K., Lee, S.I., 1982. Clinical Application of Oriental Medicine, Sungbosa, Seoul, 62, 71, 73, 130, 140, 142, 243, 268, 361, 402, 505, 539, 545.
19. Mayeux, P.R., 1997. Pathobiology of lipopolysaccharide. Journal of Toxicology and Environmental Health 51, 415-435.
20. Laskin, D.L., Pendino, K.J. 1995. Macrophages and inflammatory mediators in tissue injury. Annu Rev Pharmacol Toxicol. 35, 655-677.
21. Mojena, M., Hortelano, S., Castrillo, A., Diaz-Guerra, M.J., Garcia-Barchino, M.J., Saez, G.T., et al. 2001. Protection by nitric oxide against liver inflammatory injury in animals carrying a nitric oxide synthase-2 transgene. FASEB J. 15, 583-585.
22. Shapira, L., Soskolne, W.A., Houri, Y., Barak, V., Halabi, A., Stabholz, A., 1996. Protection against endotoxic shock and lipopolysaccharide-induced local inflammation by tetracycline: correlation with inhibition of cytokine secretion. Infect Immun. 64, 825-828.
23. MacMicking, J., Xie, Q.W., Nathan, C., 1997. Nitric oxide and macrophage function. Annu. Rev. Immunol. 15, 323-350.
24. Yui, Y., Hattori, R., Kosuga, K., Eizawa, H., Hiki, K., Kawai, C., 1991. Purification of nitric oxide synthase from rat macrophages. J. Biol .Chem. 266, 12544-12547.
25. Chang, H.K., Yang, H.Y., Lee, T.H., Shin, M.C., Lee, M.H., Shin, M.S., Kim, C.J., Kim, O.J., Hong, S.P. Cho, S., 2005. Armeniacae semen extract suppresses lipopolysaccharide-induced expressions of cyclooxygenase [correction of cycloosygenase]-2 and inducible nitric oxide synthase in mouse BV2 microglial cells. Biol. Pharm. Bull. 28, 449-454.
26. Xie, Q.W., Whisnant, R., Nathan, C., 1993. Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon g and bacterial lipopolysaccharide. J. Exp. Med. 177, 1779-1784.
27. Adams, V., Nehrhoff, B., Spate, U., Linke, A., Schulze, P.C., Baur, A., Gielen, S., Hambrecht, R., Schuler, G., 2002. Induction of iNOS expression in skeletal muscle by IL-1β and NF-kB activation: an in vitro and in vivo study. Cardiovasc. Res. 54, 95-104.
28. Munoz-Fernandez, M.A., Fernandez, M.A., Fresno, M.,1992. Synergism between tumor necrosis factor-alpha and interferon-gamma on macrophage activation for the killing of intracellular Trypanosoma cruzi through a nitric oxide-dependent mechanism. Eur. J. Immunol. 22, 301-307.
29. Harbrecht, B.G., Di Silvio, M., Demetris, A.J., Simmons, R.L., Billiar, T.R., 1994. Tumor necrosis factor-alpha regulates in vivonitric oxide synthesis and induces liver injury during endotoxemia. Hepatology 20, 1055-1060.
30. Trinchieri, G., 1998. Proinflammatory and immunoregulatory functions of interleukin-12. Int. Rev. Immunol. 16, 365-396.