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Original Article

Anti-inflammatory Effects of *Sam-chul-kun-bi-tang*

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Objective: To derive information on the efficacy of *Sam-chul-kun-bi-tang* (SKT), by evaluating its anti-inflammatory effect. SKT is a widely-used herbal formula in traditional Korean medicine. In many studies, plant-derived anti-inflammatory efficacies have been investigated for their potential inhibitory effects on lipopolysaccharide (LPS)-stimulated macrophages. This study was performed to examine the anti-inflammatory effects of SKT extract on LPS-stimulated RAW 264.7 cells.
Methods: The production of nitric oxide (NO), prostaglandin (PG)E_2, tumor necrosis factor (TNF)-α and interleukin (IL)-6 were examined in a macrophage cell line, RAW 264.7 cells, in the presence of SKT. RAW 264.7 cells were incubated with LPS 1 μg/mL and SKT for 18 hrs. The anti-inflammatory activity of SKT was investigated by carrageenan-induced paw edema in rats. The paw volume was measured at 2 and 4 hrs following carrageenan-induced paw edema in rats.
Results: SKT showed inhibitory effect on PGE_2, TNF-α and IL-6 in LPS-stimulated RAW 264.7 cells. But SKT was not inhibitory effect on NO by LPS-stimulated RAW 264.7 cells. Administration of SKT (1 g/kg) also showed a reduction in carrageenan-induced paw edema on rats.
Conclusion: These results suggest that SKT has anti-inflammatory activities in both *in vitro* and *in vivo* models.

Key Words : Anti-inflammation; *Sam-chul-kun-bi-tang*; herbal formula, lipopolysaccharide (LPS)

Introduction

Sam-chul-kun-bi-tang (SKT) is an herbal formula that is widely used in Korean traditional medicine for treatment of chronic gastritis, gastric ulcers and gastroptosis. It has been reported that SKT has pharmacological activities in processes such as immune regulation[1)] and gastroprotection[2)]. Ginseng radix, one of the constituents of SKT, has been used to prevent various diseases, including diabetes, cancer, allergy and hypertension[3)] and to treat inflammation[4)]. Atractylodis rhizoma alba, one of the constituents of SKT, has been reported to inhibit melanin biosynthesis[5)]. No previous study has empirically examined whether SKT has anti-inflammatory properties.

Inflammation is a physiological process that occurs in response to tissue or cell damage, such as that resulting from pathogenic infection or chemical irritation[6)]. Although inflammation is an important part of the body's ability to protect itself, excessive inflammation, such as that triggered by chronic inflammatory disease or cancer, can be harmful. During inflammatory processes, large amounts of the pro-inflammatory mediators, nitric oxide (NO) and

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prostaglandin E_2 (PGE_2), are generated by inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively[7]. NO production by iNOS may be induced by the microbial infection and signaling by various pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ, both of which play important roles in inflammation[8].

We examined the potential anti-inflammatory properties of SKT. As an *in vitro* model system, we used LPS-stimulated macrophages. In the present study, we investigated the anti-inflammatory effects of SKT *in vitro* by monitoring the generation of NO, PGE_2, TNF-α and IL-6. Finally, we investigated the potential anti-inflammatory effects of SKT *in vivo*, using a rat model of carrageenan-induced paw edema[9].

Materials and Methods

1. Preparation of the SKT extract

SKT was prepared according to a transitional herbal formula listed in *Dongeuibogam* (Table 1)[10]. The chopped herbs were combined in the listed ratio and extracted in distilled water at 100ºC for 2 hrs. The yield of lyophilized extract from starting crude materials was about 24.5%. The dried extract was dissolved in PBS.

2. Cell line and culture

The RAW 264.7 cell line was obtained from the American Type Culture Collection (ATCC, USA.). RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand island, NY) supplemented with 100 U/ml penicillin, 100 μg/mL streptomycin (Gibco, Grand island, NY) and 5.5% fetal bovine serum (FBS; Gibco, Grand island, NY). Cells were incubated at 37ºC, 5% CO_2 in a fully humidified condition.

3. Cell viability assay

RAW 264.7 cells were plated to 96-well plates at a density of 5×10^3 cells/well, and treated with SKT for 24 hrs. The cell counting kit-8 (CCK-8) reagent (Dojindo, Kumamoto, Japan) was added and incubated

Table 1. Composition and Content of Sam-chul-kun-bi-tang (SKT)

Herb	Content (g)
Ginseng Radix	3.750
Atractylodis Rhizoma Alba	3.750
Hoelen	3.750
Magnoliae Cortex	3.750
Aurantii Nobilis pericarpium	3.750
Crataegii Fructus	3.750
Ponciri Fructus	3.000
Paeoniae Radix	3.000
Amomi Fructus	1.875
Massa Medicata Fermentata	1.875
Hordei Fructus Germiniatus	1.875
Glycyrhizae Radix	1.875
Zingiberis Rhizoma	3.750
Zizyphi Jujubae Fructus	3.750
Total	43.500

for 4 hrs. Thereafter, absorbance was read at 450 nm, and the percentage of cell viability was calculated.

4. Measurement of NO production

RAW 264.7 cells were seeded at 2.5×10^5 cells/well (48 well plate) and incubated with LPS (1 μg/mL) for 18 hrs in the presence of various concentrations of SKT. The quantity of nitrite accumulated in the culture medium was measured as an indicator of NO production. Briefly, 50 μL of cell culture medium was mixed with 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid; Promega, Madison, WI), the mixture was incubated at room temperature for 30 min, and the absorbance was measured at 535 nm in a microplate reader (BIO-RAD, San Diego, CA). Fresh culture medium was used as a blank in every experiment. The quantities of nitrite were determined based on sodium nitrite standard curves.

5. Measurement of PGE_2 production

The RAW 264.7 cells were seeded at 2.5×10^5 cells/well (48 well plate) and incubated for 18 hrs in the presence of LPS (1 μg/mL) and various concentrations of SKT, and arachidonic acid (30 μM) was added. Following 15 min incubation, the PGE_2 concentration in the culture medium was quantified using a competitive ELISA kit (Amersham, Little Chalfont, BU) according to the manufacturer's instructions. The production of PGE_2 was measured relative to that in control cultures.

6. Measurement of inflammatory cytokines (TNF-α and IL-6) production

RAW 264.7 cells were treated as described above, and the TNF-α and IL-6 concentrations in the culture medium were quantified using an ELISA kit (R&D, Minneapolis, MN) according to the manufacturer's instructions. Absolute concentrations were obtained by comparison to standard curves on the same ELISA plates.

7. Experimental animal

4-weeks-old male Sprague-Dawley (SD) rats were purchased from Orient Incorporated (Seongnam, Korea). The animals were kept under controlled environmental conditions (22±3℃ with 12/12 hrs light/dark cycle) for one week prior to the experiment. Animals were given rodent diet and water *ad libitum*. All studies were conducted in accordance with the National Institute of Health "Guide for the Care and Use of Laboratory Animals".

8. Carrageenan-induced paw edema in rats

Edema or swelling is a cardinal sign of acute inflammation and is therefore a useful parameter to look at when testing for agents which may be active in treating acute inflammation. In this experiment, rats were divided into three groups (5 rats per group). Group 1 was the control group which was administrated vehicle (0.1% Tween 80), Group 2 was administrated SKT (1 g/kg body weight) orally for seven days prior to induction of edema, and Group 3 (positive control) was administrated indomethacin (10 mg/kg body weight; Sigma, Saint Louis, MI) orally 3 hrs prior to induced paw edema. Edema was induced by subcutaneous injection of 0.1 mL of PBS containing 1% (v/v) carrageenan (Sigma, Saint Louis, MI) into the right hind paw. Right hind paw volume was measured using a plethysmometer (Ugo-Basile Co., Italy). The paw volume was measured at 2 and 4 hrs following carrageenan-induced paw edema in rats.

9. Statistical analysis

The data are presented as mean ± S.E.M. Data were analyzed using the paired Student's t-test. The level of significance was set at $p<0.05$.

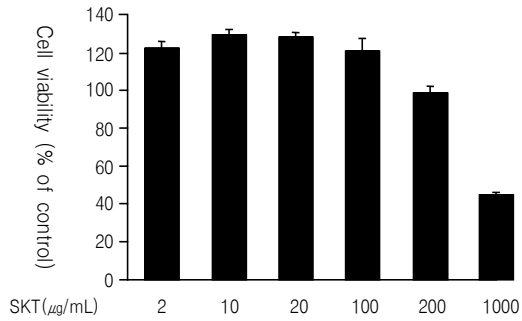

Fig. 1. Effects of *Sam-chul-kun-bi-tang* (SKT) on viability of RAW 264.7 cells.
Cells were treated with various concentrations of SKT (2-1000 μg/ml) for 24 hrs, and cell viability was determined using the CCK-8 reagent. Data are given as mean ± S.E.M. from triplicate independent experiments.

Results

1. Cell viability

We evaluated the 24 hrs toxicity of various concentrations of SKT, using the CCK-8 reagent. As shown in Fig. 1, cell viability was not significantly altered by SKT up to 200 μg/mL. Thus, non-toxic concentrations of SKT were used for all subsequent experiments.

2. Inhibition of PGE_2 production but not NO in LPS-stimulated RAW 264.7 cells

We investigated the effect of SKT on two inflammatory mediators, PGE_2 and NO, in LPS-stimulated RAW 264.7 cells. As shown in Fig. 2, the levels of PGE_2 increased in the culture media of LPS-stimulated RAW 264.7 cells but this increase was concentration-dependently inhibited by addition of SKT. SKT (200 μg/mL) significantly inhibited PGE_2 production by 57% ($p<0.05$ compared to LPS). In contrast, the levels of nitrite were not significantly different for LPS-stimulated RAW 264.7 cultured with and without SKT (data not shown).

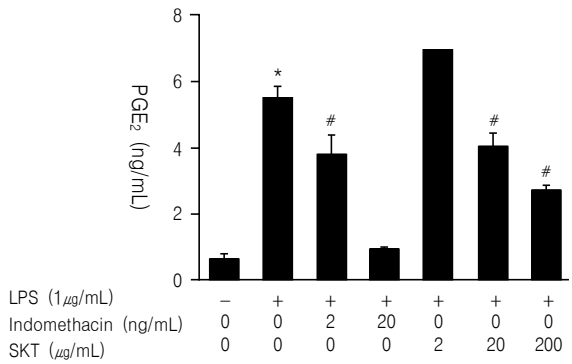

Fig. 2. Effects of *Sam-chul-kun-bi-tang* (SKT) on LPS-stimulated PGE_2 production in RAW 264.7 cells. Cells were treated with LPS (1 μg/mL) and cultured for 18 hrs with or without SKT. The PGE_2 concentrations in cell culture supernatants were measured as described in Materials and Methods. Data are presented as mean ± S.E.M. and are representative of triplicate experiments.
* $p<0.05$ compared to control (LPS-free). # $p<0.05$ compared to cells cultured with LPS.

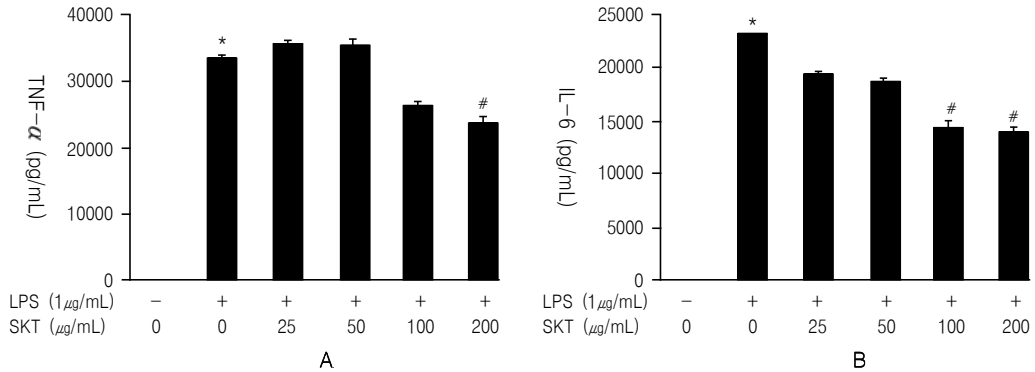

Fig. 3. Effects of *Sam-chul-kun-bi-tang* (SKT) on LPS-stimulated TNF-α (A) and IL-6 (B) production in RAW 264.7 cells. Cells were treated with LPS (1 μg/mL) and cultured for 18 hrs with or without SKT. The TNF-α and IL-6 concentrations in cell culture supernatants were measured as described in Materials and Methods. Data are presented as mean ± S.E.M. and are representative of triplicate experiments.

* $p<0.05$ compared to control (LPS-free). # $p<0.05$ compared to cells cultured with LPS.

3. Inhibition of TNF-α and IL-6 production in LPS-stimulated RAW 264.7 cells

To examine the effects of SKT on inflammatory cytokines, we monitored TNF-α and IL-6 production in LPS-stimulated RAW 264.7 cells. When RAW 264.7 cells were induced with LPS (1 μg/mL) for 18 hrs, the levels of TNF-α and IL-6 in the cultured media increased. SKT concentration-dependently inhibited this effect for both cytokines (Fig. 3A and B). SKT 200 μg/mL significantly inhibited TNF-α and IL-6 production by 34% and 43% ($p<0.05$ compared to LPS), respectively.

4. Carrageenan-induced paw edema in rats

The anti-inflammatory effect of SKT was evaluated *in vivo*, using carrageenan-induced paw edema in SD rats. Our results revealed that pre-administration of SKT reduced the degree of paw edema seen following

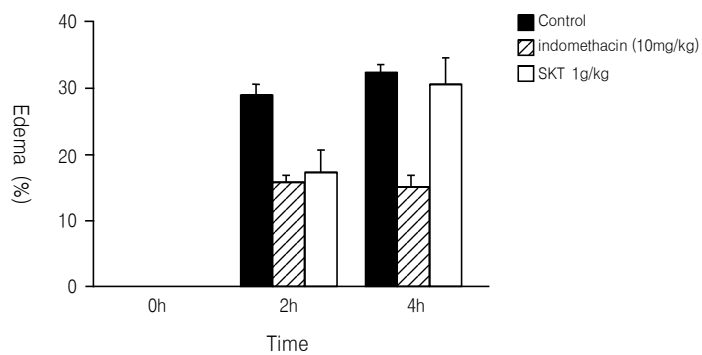

Fig. 4. Effects of *Sam-chul-kun-bi-tang* (SKT) on carrageenan-induced paw edema in rats. The control group (0.1 % Tween 80) and SKT group (1 g/kg) were orally administered for seven days prior to the inception of carrageenan-induced paw edema. The indomethacin group (10 mg/kg) was orally administered for 3 hrs prior to the inception of carrageenan-induced paw edema. The paw volume was measured as described in Materials and Methods. Data are presented as mean ± S.E.M (5 rats per group).

carrageenan injection (Fig. 4). SKT inhibited the paw edema by 40% and 8% compared to control at 2 and 4 hrs, respectively.

Discussion

Various studies have shown that certain traditional herbal medicines have therapeutic effects against inflammation. SKT has been used to treat inflammatory stomach diseases, such as chronic gastritis and gastric ulcer[1,2]. In this study, we demonstrate that SKT significantly inhibited the production of PGE_2 and pro-inflammatory cytokines, such as TNF-α and IL-6 in LPS-stimulated RAW 264.7 cells. We further show that pre-administration of SKT reduced the extent of carrageenan-induced paw edema in rats.

Macrophages are the major cell of the mononuclear phagocyte system. During inflammation, macrophages have three major functions: antigen presentation, phagocytosis, and immunomodulation through the production of various cytokines and growth factors[11]. LPS is an endotoxin, which induces septic shock and stimulates the production of inflammatory mediators such as NO, PGE_2, TNF-α and IL-6[12,13]. Although our results revealed that SKT did not affect the production of NO, which is an important mediator of acute and chronic inflammation[14,15] treatments with SKT significantly inhibited the production of PGE_2 in LPS-stimulated RAW 264.7 cells, which were used as an *in vitro* model of inflammation (Fig. 2). PGE_2, which is one of the strongest inflammatory mediators involved in the inflammatory response[16], is transformed from arachidonic acid via the COX-2 catalytic reaction. Therefore, the newly developed PGE_2 inhibitors share a common therapeutic action on inflammation.

TNF-α is known as an inflammatory factor. It can induce the release of pro-inflammatory cytokine (e.g., IL-1β and IL-6). IL-6 plays important roles in immunological and inflammatory responses[17]. TNF-α and LPS have both been shown to induce IL-6 gene expression and promote IL-6 secretion via release of the nuclear binding protein NF-κB[18,19]. The ability of an agent to inhibit LPS-stimulated increases in TNF-α and IL-6 production has been used to assess the potential anti-inflammatory effects of drug candidates[20] and traditional (herbal) medicines[21]. Our experiments further revealed that the productions of TNF-α and IL-6 in LPS-stimulated RAW 264.7 cells were inhibited by SKT (Fig. 3A and B). Therefore, our finding that SKT can block LPS-stimulated increases in both TNF-α and IL-6 suggests that SKT has anti-inflammatory effect.

Carrageenan-induced inflammation in the rat paw, which is a classical model of edema formation and hyperalgesia, has been extensively used in the development of nonsteroidal anti-inflammatory drugs and selective COX-2 inhibitors[22]. This model allows quantitative assessment of both inflammation and the formation of chemical mediators such as cytokines[23]. This study exhibits inhibition of carrageenan-induced paw edema by SKT and its activity was comparable to indomethacin, a known cyclooxygenase inhibitor (2 hrs following carrageenan-induced paw edema in rats) (Fig 4).

Conclusion

In conclusion, our results demonstrate that SKT appears to confer anti-inflammatory effects *in vitro* and *in vivo*. We are currently trying to identify the active components of SKT, and to gain an understanding of the mechanisms underlying its anti-inflammatory function.

Acknowledgments

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