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Original Article / 원저

Anti-Inflammatory Effects of *Tongbi-san*(通痺散) Extract on RAW264.7 Macrophages

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통비산(通痺散) 열수추출물의 항염증반응 및 항산화활성에 대한 연구

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Abstract

Objectives : This study is to investigate the anti-inflammatory and anti-oxidant effects of *Tongbi-san* extract (TS) on RAW264.7 macrophages using by cell cytotoxicity, Nitric Oxide (NO) and Prostaglandin E_2 (PGE_2) production and 1,1-diphenyl-2-picryl ghdrazyl (DPPH) free radical scavenging capability.

Methods : Cell cytotoxicity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The production of NO was measured by Griess assay. The production of PGE_2 was measured by immunoassay. And the anti-oxidant activity was measured by the DPPH method.

Results : TS did not increased significantly compared to the TS untreated group in the cell cytotoxicity. TS inhibited NO and PGE_2 production in lipopolysaccharide-stimulated RAW 264.7 cells. TS had the DPPH free radical scavenging capability.

Conclusion : The anti-inflammtory and anti-oxidant effects of TS may be use for a treatment of anti-inflammatory diseases.

Key words : Anti-inflammation; Anti-oxidant activity; Nitric Oxide; Prostaglandin E_2; reactive oxygen species; *Tongbi-san*

Ⅰ. Introduction

Traditional medicine is an important natural source of phytochemical compounds with substantial therapeutic effects and represents the primary health resource to many people[1]. World Health Organization (WHO) estimates that 80 % of people indeveloping countries use traditional medicine as primary healthcare[2]. Therefore, it is important to assess and validate the traditional effects of plants to assure people that consume them[3].

Inflammation is a local, protective response of the immune system. Excessive inflammatory responses can be harmful, as in diseases such as rheumatoid arthritis, Alzheimer's disease and septic shock syndrome[4]. Lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, stimulates macrophages to produce pro-inflammatory mediators such as tumor necrosis factor alpha, interleukin-6, and inducible nitric oxide synthase, which trigger a cascade responsible for the inflammatory response[5]. Antioxidants can protect against the damage caused by free radicals that have been implicated in the etiology of large number of major diseases[6].

Nitric oxide (NO) is synthesized from amino acid, arginine, by nitric oxide synthase (NOS). NO plays an important role as a vasodilator, neurotransmitter and in the immunological system as a defense against tumor cells, parasites and bacteria[7]. However, NO production is increased by the inducible isoform of NOS (iNOS), subsequently, brings about cytotoxicity and tissue damage[8]. Therefore, much attention has focused on how to decease the NO production generated by iNOS.

In addition, cyclooxygenase 2 (COX-2) is the rate limiting enzyme and responsible for the catalysis of prostaglandin E_2 (PGE_2) from arachidonic acid[9]. Chang et al. noted that the induction of COX-2 activity and subsequent generation of PGE_2 are closely related to the NO production[10]. Thus, reduce the levels of PGE_2 and the levels of COX-2 may be an effective strategy for inhibiting the inflammation and carcinogenesis.

Tongbi-san (TS) has been used in pain control, improve blood circulation, and mediate injury healing. It is consistently used in the clinical treatment of pain and bone injury[11]. In the present study, protective effect of Tongbi-san on the effects of anti-inflammation and and-oxidation by modulation of nitric oxide and PGE_2 production in LPS-stimulated RAW264.7 macrophages were investigated.

Ⅱ. Materials & Methods

1. Cell culture

Cells of the murine macrophage RAW 264.7 were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were cultrued in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal

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bovine serum (FBS) (Gibco BRL) at 37℃ in 5% CO_2, 95% O_2 in a humidified cell incubator. Cells were plated in culture dish (Corning Incorporated, Corning, NY, USA) at a density of 1×10^6 cells per dish, and the media was changed once every 2 days.

2. Preparation of extract

Tongbi-san (TS) were obtained from Semyung Korean medical hospital (Chungbuk, Korea). The procedure in brief is as follow: each medicinal plants were performed reflux extraction with distilled water (D.W) for 3 h at 100℃. Filtration and evaporation were performed with rotary vacuum evaporator (N-N series, EYELA, Japan) at 60℃. The solution was freeze dried for 24 h at 80℃ and lyophilized to yield. The composition and dosage of *Tongbi-san* (TS) are epitomized in Table 1.

Table 1. The Composition of *Tongbi-san* (TS)

Herbal medicine name	Dosage (g)
Gastrodiae Rhizoma 天麻	24
Araliae Continentalis Radix 獨活	16
Angelicae Tenuissimae Radix 蕘本	16
Angelica Gigantis Radix 當歸	16
Cnidii Rhizoma 川芎	16
Atractylodis Rhizoma Alba 白朮	16
Total	104g

3. MTT cytotoxicity assay

Cell viability was determined by the MTT assay kit using as per the manufactures protocol. Cells were cultured in 96 well plates. Experimental groups are exposed to TS at final concentrations of 50, 100, 200 and 400 ㎍/㎖ for 24 hrs, and saline of an equal volume was added to untreated group. Ten ㎖ of the MTT labeling reagent was then added to each well, and the plates were incubated for 4 h. After the cells were incubated in 100 ㎖ of the solubilization solution for 12 h, the absorbance was measured with a microtiter plate reader (Bio-Tek, Winooski, VT, USA) at a test wavelength of 595 ㎚ with a reference wavelength of 690 ㎚. The optical density (O.D.) was calculated as the result of the subtraction of the absorbance at the reference wavelength from that of the test wavelength. Percent viability was calculated as (O.D. of drug-treated sample/control O.D.) × 100.

4. NO assay

The concentration of NO in the culture supernatants was determined by measuring nitrite, a major stable product of NO, using the Griess reagent. Briefly, Cells were plated onto 24-well plates and pretreated with the various concentrations of TS 1 h prior to stimulation with 1 ㎍/㎖ of LPS for 24 h. Supernatant samples were mixed with equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylene diamine dihydro chloride in 5% phosphoric acid) and the incubated at room temperature for 10 min. The absorbance was measured at 540 ㎚ on a microplate reader (Thermo electron corporation, Marietta, OH).

5. Measurement of PGE2

Enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) was used to measure PGE_2 production according to the

manufacturer's instructions. Cells were plated in 24-well plates and pretreated with the indicated concentrations of TS 1 h prior to stimulation with 1 ㎍/㎖ LPS for 24 h. One hundred microliters of culture media were collected for the determination of PGE_2 concentrations by ELISA according to the manufacturer's instructions.

6. Assessment of DPPH radical scavenging activity

The DPPH radical scavenging activity was mesured according to previous studies with a few modifications. Briefly, 2 ㎖ of 0.2 mM methanolic solution of DPPH radicals were added to 2 ㎖ of water-solution of TS at various concentrations. The absorbance of the mixture was measured at 517 nm after 30 min of incubation at 37℃ in the dark. Ascobic acid was used as the control and distilled water as the blank. The scavenging effect was calculated according to the following equation: Scavenging rate %=(1-As/A0)×100%, where as is the absorbance obtained for a sample and A0, the absorbance of the blank.

7. Statistical analysis

Statistical analysis was performed using Student's t-test (SPSS ver 12.0) and the results were expressed as mean ± S.E.M. Differences were considered significant for $p < 0.05$.

Ⅲ. Results

1. Cell viability assay in RAW 264.7 macrophages

In order to find out the concentration at which the cytotoxic effect of TS on the RAW 264.7 cell line become evident, cells were cultured with TS at final concentrations of 50 ㎍/㎖, 100 ㎍/㎖, 200 ㎍/㎖, and 400 ㎍/㎖ for 24 hrs, and MTT assays were carried out, with cells cultured in TS-free media as the control. The viabilities of cells incubated with TS at concentrations of 50 ㎍/㎖, 100 ㎍/㎖, 200 ㎍/㎖ and 400 ㎍/㎖ were 99.41 ± 1.60 %, 97.47 ± 1.87 %, 94.39 ± 2.88 % and 88.50 ± 5.71 % of the control (100 ± 1.63 %) value respectively. As shown in Fig. 1, TS, at concentrations 50-400 ㎍/㎖, showed no obious cytotoxicity on RAW 264.7 cells.

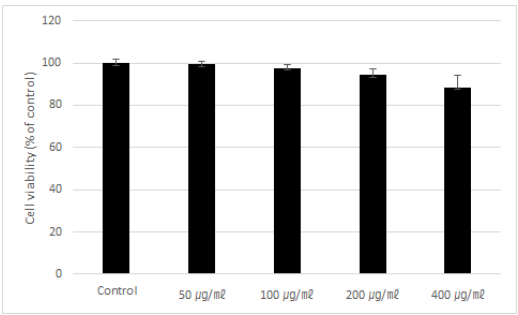

Fig. 1. Cytotoxic effects of *Tongbi-san* aqueous extract (TS). RAW 264.7 cells were incubated with TS at various concentrations (0-400 ㎍/㎖) prior to the determination of cellular viability through MTT assay. Results were represented as mean ± standard error.

2. Effect of *Tongbi-san* (TS) on NO release in LPS-stimulated RAW 264.7 macrophages

To determine the effects of TS on NO production in RAW 264.7 cells, the cells were pre-incubated with various concentrations of TS for 1 h and then stimulated with 1 ㎍/㎖ of LPS for 24 h. The control group was not treated with

LPS or TS. Supernatant from cell culture media was collected, and NO levels were determined with the Griess assay. TS was found to inhibit LPS-induced NO productions in a dose-dependent manner. The NO production of TS at concentrations of 50 ㎍/㎖, 100 ㎍/㎖, 200 ㎍/㎖ and 400 ㎍/㎖ were 98.46 ± 3.35 %, 93.85 ± 7.02 %, 90.49 ± 2.14 % and 78.01 ± 3.95 % of the control (100 ± 4.87 %) value respectively. TS at 200 ㎍/㎖ and 400 ㎍/㎖ significantly inhibited NO production(Fig. 2).

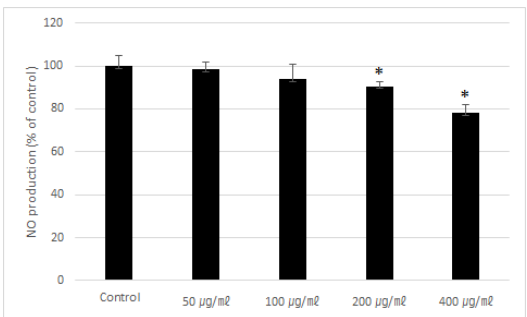

Fig. 2. Effects of *Tongbi-san* aqueos extract (TS) on the nitric oxide (NO) production in RAW 264.7 cells. Control : treated with LPS (1 ㎍/㎖) ; 50-400 : treated with LPS and TS (50-400 ㎍/㎖). *represents $p<0.05$ compared to the Control group.

3. Effect of *Tongbi-san* (TS) on PGE2 release in LPS-stimulated RAW 264.7 macrophages

To determine the effects of TS on PGE_2 production in RAW 264.7 cells, the cells were pre-incubated with various concentrations of TS for 1 h and then stimulated with 1 ㎍/㎖ of LPS for 24 h. The control group was not treated with LPS or TS. Supernatant from cell culture media was collected, and PGE_2 levels were determined with the EIA kit. TS was found to inhibit LPS-induced PGE_2 productions in a dose-dependent manner. The PGE_2 production of TS at concentrations of 50 ㎍/㎖, 100 ㎍/㎖, 200 ㎍/㎖ and 400 ㎍/㎖ were 739.81 ± 11.45 pg/well, 732.92 ± 21.11 pg/well, 706.58 ± 14.92 pg/well, 675.25 ± 13.90 pg/well of the control (742.24 ± 11.63 pg/well) value respectively. TS at 200 ㎍/㎖ and 400 ㎍/㎖ significantly inhibited PGE_2 production(Fig. 3).

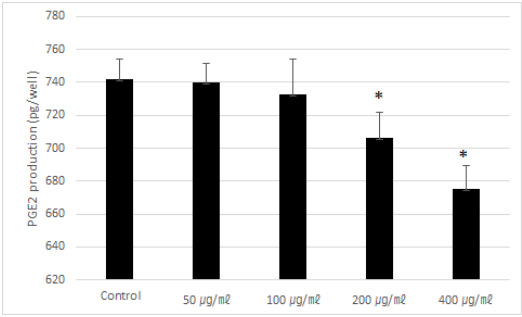

Fig. 3. Effects of *Tongbi-san* aqueos extract (TS) on the prostaglandin E2 (PGE2) production in RAW 264.7 cells. Control : treated with LPS (1 ㎍/㎖) ; 50-400 : treated with LPS and TS (50-400 ㎍/㎖). *represents $p<0.05$ compared to the Control group.

4. Effect of *Tongbi-san* (TS) on DPPH radical scavenging activity

Scavenging of DPPH radicals is the basis of a common antioxidant assay. Antioxidants can protect against the damage caused by free radicals that have been implicated in the etiology of large number of major diseases. TS displayed concentration dependent radical scavenging effects. TS at concentrations of 50 ㎍/㎖, 100 ㎍/㎖, 200 ㎍/㎖ and 400 ㎍/㎖ were 15.34 ± 1.98 %,

27.86 ± 2.03 %, 47.03 ± 1.54 % and 75.27 ± 1.75 %(Fig. 4).

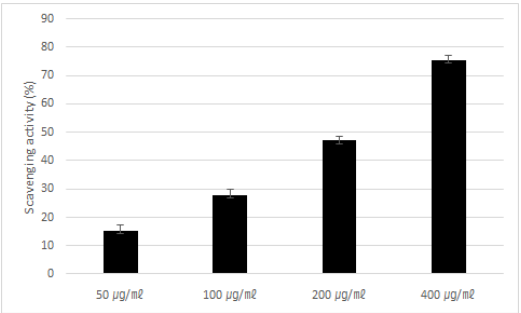

Fig. 4. DPPH free radical scavenging capability of *Tongbi-san* aqueos extract (TS). The absorbance of the TS (50-400 μg/㎖) was measured at 517 ㎚. Results were represented as mean ± standard error.

Ⅳ. Discussion

The purpose of this study is to investigate the effects of TS on the production of NO and PGE_2 induced by LPS-stimulated RAW 264.7 cells and DPPH free radical scavenging capability.

It is well known that Korean herbal medicine, TS, which is comprised six herbs of *Gastrodiae Rhizoma, Araliae Continentalis Radix, Angelicae Tenuissimae Radix, Angelica Gigantis Radix, Cnidii Rhizoma and Atractylodis Rhizoma Alba*[11]. These herbs are effective for the treatment of inflammation, hyperlipidemia, arteriosclerosis, and gynecological disease. However, there was no research about the anti-inflammatory and anti-oxidant effects of TS[12-15].

In this study, we used TS as the potent NO and PGE_2 production inhibitor to investigate the involvement of anti-inflammation and anti-oxidant effect. The TS reduced NO production and PGE_2 production induced by the induction of LPS, and increased the radical scavenging activity. The radical scavenging activity of TS is and indication that it is a potential natural antioxidant.

It is well known that chronic low grade inflammation is involved in a range of pathophysiologic metabolic disorders and diseases such as obesity, insulin resistance, cardiovascular disease and cancer. In obesity, low grade inflammation underlies with an aberrant infiltration of activated macrophages in dysfunctional adipose tissue. This systemic process involves activation of intracellular signaling pathways able to release arrested NF-κB by the inhibitor of κB (IκB) proteins, allowing its nuclear translocation, and development of the expression of genes (iNOS/NO, COX-2/PGE_2) involved in inflammation as well as cytokines, chemokines and grow factors. This process plays an important function in the control of apoptosis, oxidative/nitrosative stress and proliferation[3]. Furthermore, activation of this signaling pathway is associated with persistent release of pro-inflammatory mediators, which drive to insulin resistance and chronic diseases[16-18]. Hence, alternative phytochemical compounds targeting intracellular NF-κB signaling pathway could contribute to the anti-inflammatory process and inhibition of its associated pathologies[3].

Inflammation is a complex process regulated by a variety of immune cells and effector molecules. NO, PGE_2, and pro-inflammatory cytokines are important mediators of macrophage-mediated inflammation[19]. Therefore, the inhibition of these mediators with pharmacological modulators may

be an effective therapeutic strategy for preventing inflammatory reactions and diseases[20].

Macrophages play critical roles in immune reactions, allergy, and inflammation[21]. These cells induce inflammatory reactions, and initiate and maintain specific immune responses by releasing different types of cytokines. LPS, a comonent of the gram-negative bacterial cell wall, has often been used in inflammatory response because it can activate macrophages[22].

In general, NO plays an important role in the antitumour, antivirus replication and other diseases[23], the overproduction of NO is harmful to the host, leading to rheumatoid arthritis[24] and allograft rejection[25]. NO production from macrophages can be induced by inflammatory cytokines or bacterial products, including LPS, IFN-γ, or TNF-α[26].

PGE_2 is considered the one of the strongest inflammatory mediators in inflammatory response. It was transformed from arachidonic acid via the cyclooxygenase-2 (COX-2) catalytic reaction. Nonsteroidal anti-inflammatory drugs (NSAIDs), which were used widely in current clinical, play their antipyretic, anti-inflammatory and analgesic effects through the inhibition of COX activity and the reduction of inflammatory mediator production such as PGE_2[27].

In no stimulated cells, ROS are generated by normal metabolism and include hydrogen peroxide, hydroxylradical and superoxide anions radicals. In basal ROS production, NADP+/NADPH oxidase complex maintains redox homeostasis, but in overproduction, numerous detoxifying/antioxidative enzymatic systems are developed such as superoxide dismutase, catalase and glutathione peroxidase and hemeoxigenase-1. However, an imbalance between ROS production and inadequate anti-oxidant mechanism results in an oxidative stress state. Several researchers have reported that anti-oxidant properties of polyphenols can produce their chemo-preventive effects through NF-κB and nuclear factor E_2 related factor (Nrf2)/anti-oxidant response element (ARE) pathways activation[28].

Oxidative stress is an important factor in the genesis of most pathologies, ranging from cancer to cardiovascular and degenerative diseases[29]. In order to protect the body against the consequences of oxidative stress, an efficacious approach consists in improving the anti-oxidant nutrition. Anti-oxidants from natural sources have a higher bioavailability and therefore higher protective efficacy than synthetic anti-oxidants[30].

Free radicals are chemical species with one or two unpaired electrons in their outermost layer, which can be created in a multiple ways. They can be exogenic or endogenic. A lack of anti-oxidant or an overproduction in free radicals cab lead to an imbalance between the oxidant and anti-oxidant system. One of the most significant factors in the production of free radicals is oxidative stress[31]. Oxidative stress is involved in a several illnesses, including diabetes[32], atherosclereosis, Alzheimer's disease, Parkinson's disease, glaucoma and age-related macular degeneration[33]. The provision of anti-oxidants through diet or herb-medicine is a simple means to reduce the development of illnesses brought on by oxidative stress[34].

TS has effects on reinforcing kidney, improving

circulation, reduce bleeding, air wound healing, etc[35]. In the clinical setting, TS is consistently used for the treatment of inflammation as well as pain control. Under these influences, modern pharmacological studies mainly focus on the anti-inflammatory effect and anti-oxidant effect.

Ⅴ. Conclusion

1. This study is to investigate the anti-inflammatory and anti-oxidant effects of TS extract on RAW264.7 macrophages using by cell cytotoxicity, NO and PGE_2 production and DPPH free radical scavenging capability.
2. TS did not increased significantly compared to the untreated group in the cell cytotoxicity.
3. TS inhibited NO and PGE_2 production in lipopolysaccharide-stimulated RAW264.7 cells.
4. TS had the DPPH free radical scavenging capability.
5. The anti-inflammtory and anti-oxidant effects of TS may be use for a treatment of inflammatory diseases.

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