Journal of Korean Medicine Rehabilitation Vol. 32 No. 4, October 2022
pISSN 1229-1854 eISSN 2288-4114
https://doi.org/10.18325/jkmr.2022.32.4.9

JKMR
Original Article

내탁백렴산 추출물의 항염증 효능 연구

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Anti-inflammatory Effects of *Naetakbaekryeom-san*

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본 연구는 보건복지부의 재원으로 한국보건산업진흥원의 보건의료기술연구개발사업 지원에 의하여 이루어진 것임(과제고유번호: HI21C1924).

RECEIVED June 17, 2022
REVISED July 8, 2022
ACCEPTED July 12, 2022

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Objectives This study was conducted to confirm the anti-inflammatory effect of *Naetakbaekryeom-san* (NTB), and whether it could be another treatment for inflammatory diseases.
Methods The NTB water extract was extracted with hot water at 100℃ for 2 hours, concentrated at 80℃ under reduced pressure, and used. After 2 hours of pretreatment with NTB and positive control Bay11-7082, nitric oxide (NO), inducible NO synthase (iNOS), interleukin (IL)-6, IL-1β, tumor necrosis factor alpha (TNF-α) were measured in RAW264.7 cells activated with lipopolysaccharides (LPS) 500 ng/mL. After 2 hours of pretreatment with NTB, the anti-inflammatory effect of NTB was evaluated by measuring nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in RAW264.7 cells and 293T cells activated with phorbol 12-myristate 13-acetic acid (PMA) 30 ng/mL.
Results In RAW264.7 cells activated with LPS, NTB at concentrations of 0.1, 0.3, and 1.0 mg/mL showed no cytotoxicity, significantly inhibited NO production and inhibition of iNOS expression. TNF-α cytokine levels was not regulated, but NTB at each concentration inhibited the production of IL-1β and IL-6, and the effect was higher than that of the positive control Bay11-7082 (20 μM). In PMA-activated RAW264.7 cells and 293T cells, each concentration of NBT decreased the NF-κB transcriptional activity, with the greatest decrease at 1 mg/mL.
Conclusions These results demonstrated the anti-inflammatory effect of NTB water extracts, but further studies such as comparison of anti-inflammatory effects and antioxidant effects by NTB component, comparison of effects according to extraction solvents, and clinical studies are needed. **(J Korean Med Rehabil 2022;32(4):9-18)**

Key words *Naetakbaekryeom-san*, Anti-inflammatory, Herbal medicine

Introduction

The inflammatory response, which generates and heals tissues following noxious stimuli, is a defense mechanism in the body, and local symptoms include redness, fever, edema, pain, and loss of function. In tissues with acute inflammation, the blood vessels expand in response to chemical mediators, blood flow increases, and white blood

cells aggregate to remove foreign substances and necrotic material from the tissue[1-3]. If inflammation is not controlled and continues for a long time, it becomes chronic leading to damage to normal tissues and various inflammatory diseases[4-6].

Currently, steroids or nonsteroidal anti-inflammatory drugs (NSAIDs) are mainly used to suppress excessive inflammation. However, side effects such as peptic ulcer and bleeding are frequently reported following the use of NSAIDs[7]. In addition, side effects such as increased risk of infection, osteoporosis, and diabetes have been reported following the use of steroids[8]. Research to reduce the occurrence of these side effects is ongoing, and attempts are being made to treat inflammation using Korean Medicine[8,9].

Naetakbaekryeom-san (NTB) is a traditional Korean herbal medicine described in Man-Byeong-Hoi-Chun, that is composed of *Paeoniae Radix, Angelicae Gigantis Radix, Forsythiae Fructus, Angelicae Dahuricae Radix, Ampelopsis Radix, Trichosanthis Fructus, Scutellariae Radix, Cnidii Rhizoma, Trichosanthis Radix, Olibanum, Saposhnikoviae Radix, Platycodi Radix, Bupleuri Radix, Tribuli Fructus*, and *Glycyrrhizae Radix*[10,11]. Although studies have reported the anti-inflammatory effects of each of these herbs[12-26], the anti-inflammatory effects of NTB, a complex herbal medicine, have not been studied. Therefore, we conducted a study on the anti-inflammatory efficacy of NTB water extract to determine whether NTB could have therapeutic potential for the treatment of inflammation.

Inflammation was induced using lipopolysaccharides (LPS) and phorbol 12-myristate 13-acetate (PMA) in RAW264.7, a murine macrophage cell line that plays an important role in body defense by producing various cytokines during inflammatory reactions[27] and 293T cells, and cell viability was measured after treatment with NTB. In addition, the level of nitric oxide (NO) and inducible NO synthase (iNOS), which are inflammatory mediators, and the inflammatory cytokines, interleukin (IL)-6, tumor necrosis factor alpha (TNF-α) and IL-1β, were measured. The levels of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a transcriptional regulator of inflammation-related substances such as iNOS and TNF-α[27] were also measured.

Materials and Methods»»»

1. Preparation of NTB

The fifteen herbal medicines forming NTB - *Paeoniae Radix, Angelicae Gigantis Radix, Angelicae Dahuricae Radix*, Cnidii Rhizoma, Trichosanthis Radix were purchased from Seonil saeng yak (Hongcheon, Korea). *Forsythiae Fructus, Trichosanthis Fructus, Saposhnikoviae Radix* were purchased from CK Pharm (Seoul, Korea). *Ampelopsis Radix, Scutellariae Radix, Bupleuri Radix, Tribuli Fructus* were purchased from Humanherb (Daegu, Korea). *Olibanum* was purchased from Nanumherb (Yeongcheon, Korea). *Platycodi Radix* was purchased from Cypharm (Jangseong, Korea). *Glycyrrhizae Radix* was purchased from Poongsanpharm (Andong, Korea), respectively. The NTB composition was in accordance with Man-Byeong-Hoi-Chun[11], and the composition and dose of 1 pack are shown in Table I.

The herbal composition of NTB was extracted with hot water at 100°C for 2 hours in a automatic non-pressure herbal extractor (KS-220L, KYUNGSEO E&P, Incheon, Korea). The water extract of NTB was concentrated using a rotary vacuum evaporator (N1000SWD; EYELA, Tokyo, Japan) at 80°C under reduced pressure. A stock solution of NTB at a concentration of 10 mg/mL was prepared in phosphate buffered saline and filtered using a sterile syringe filter (Pall Life Science, Port Washington, NY, USA) with a pore size of 0.22 μM.

2. Reagents

Dimethyl sulfoxide, Bay11-7082, Lipopolysaccharide, Griess Reagent, Celecoxib, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), PMA were purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA). Dulbecco's phosphate-buffered saline, Dulbecco's modified Eagle's

Table I. The Herbal Composition of *Naetakbaekryeom-san*

Latin name	Scientific name	Family	Amount (g)
Paeoniae Radix	*Paeoniae lactiflora pallas*	Ranunculaceae	4
Angelicae Gigantis Radix	*Angelica gigas nakai*	Umbelliferae	4
Forsythiae Fructus	*Forsythia viridissima Lindley*	Oleaceae	4
Angelicae Dahuricae Radix	*Angelica dahurica Bentham et Hooker*	Umbelliferae	3.2
Ampelopsis Radix	*Ampelopsis japonica (Thunb.) Makino*	Vitaceae	3.2
Trichosanthis Fructus	*Trichosanthes kirilowii Maxim.*	Cucurbitaceae	3.2
Scutellariae Radix	*Scutellaria baicalensis Georgi*	Labiatae	3.2
Cnidii Rhizoma	*Cnidium officinale Makino*	Umbelliferae	2.8
Trichosanthis Radix	*Trichosanthes kirilowii Maxim.*	Cucurbitaceae	2.8
Olibanum	*Boswellia carterii Birdwood*	Burseraceae	2.8
Saposhnikoviae Radix	*Saposhnikovia divaricata Schischkin*	Umbelliferae	2
Platycodi Radix	*Platycodon grandiflorum A. (Jacq) DC.*	Campanulaceae	2
Bupleuri Radix	*Bupleurum falcatum Linne*	Umbelliferae	2
Tribuli Fructus	*Tribulus terrestris L.*	Zygophyllaceae	1.6
Glycyrrhizae Radix	*Glycyrrhiza uralensis Fisch.*	Leguminosae	1.6

medium were purchased from Welgene Inc. (Gyeongsan, Korea). Fetal bovine serum, penicillin/streptomycin were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Lysis buffer was purchased from Promega (Madison, WI, USA). Western Bright^{TM} ECL reagent was purchased from Advansta Inc. (San Jose, CA, USA). QUANTI-Blue solution was purchased from Invitrogen (Waltham, MA, USA), respectively.

3. Instruments used in the experiment

Automatic non-pressure herbal medicine extractor (KS-220L) was purchased from KYUNGSEO E&P. Rotary vacuum evaporator (N1000SWD) was purchased from EYELA. Sterile syringe filter was purchased from Pall Life Science. Shaking incubator was purchased from Daewon Science, Inc. (Bucheon, Korea). A microplate reader was purchased from BioTek Instruments, Inc. (Winooski, VT, USA). Microscope was purchased Nikon (Tokyo, Japan). Polyvinylidene flouride membrane was purchased from Millipore (Burlington, MA, USA). The primary antibodies specific to iNOS (1:1,000) and β-actin (1:1,000) were purchased from Santa-Cruz Biotechnology (Dallas, TX, USA). Horseradish peroxidase-conjugated secondary antibodies was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). Enzyme-linked immunosorbent assay (ELISA) kits IL-1β, IL-6 were purchased from R&D system (Minneapolis, MN, USA), and TNF-α was purchased from Biolegend (San Diego, CA, USA). ELISA microplatereader (ELx808) was purchased from BioTek Instruments, Inc. Poly-D-lysine was purchased from Sigma-Aldrich Co., Ltd.. pNF-κB-SEAP reporter plasmid was purchased from Clontech Laboratories (Santa Clara County, CA, USA). HilyMax was purchased from Dojindomolecular technologies, Inc. (Rockville, MD, USA). C300 was purchased from Azure Biosystems, Inc. (Dublin, CA, USA), respectively.

4. Cell cultures

RAW264.7 macrophage cells and 293T human kidney epithelial cells were obtained from American Type Culture Collection (Manassas, VA, USA) and the cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Welgene) containing 10% fetal bovine serum and 1% penicillin-streptomycin and at a 37°C incubator with 5% CO_2.

5. Test of NTB safety by cytotoxicity assay

Both RAW264.7 cells and 293T cells were used for cytotoxicity test using MTT. The cells seeded into 96-well plates (1×10^5 cells/well) were cultivated in a 37°C incubator overnight and then treated with NTB at concentrations of 1.0, 0.3, and 0.1 mg/mL for 24 hours. The cells were incubated with MTT solution (10 μL) in a 37°C incubator for 4 hours. dimethyl sulfoxide (150 μL) was added into 96-well plates after removing the supernatant. A microplate reader was used to measure absorbance at 570 nm. The images of cells treated with NTB overnight were taken under the microscope.

6. NO assay

RAW264.7 cells were seeded into a 48-well plate (1×10^5 cells/well) and cultured in a 37°C incubator overnight. The cells were treated with NTB at concentrations of 1.0, 0.3, and 0.1 mg/mL or positive control Bay11-7082 (20 μM) for 2 hours, and then treated with LPS at 500 ng/mL for 24 hours. Then supernatants (100 μL) were collected and reacted with Griess Reagent (100 μL) into a 96-well plate for 30 minutes at room temperature. The absorbance was measured at 570 nm using with a microplate reader.

7. Western blot analysis

RAW264.7 cells were seeded into 6-well plates (8×10^5 cells/well) and treated with NTB at concentrations of 1.0, 0.3, and 0.1 mg/mL for 2 hours. Bay11-7082 (20 μM) and celecoxib (20 μM) were used as positive controls. And then the cells were incubated with LPS at 200 ng/mL for 24 hours. To detect iNOS, the same amount of proteins (20 μg) extracted by using lysis buffer was analyzed by 10% sodium dodecyl sulfate-poiyacrylmide gel eletrophoresis and then electro-transferred to polyvinylidene fluoride membrane. After blocking the non-specific binding proteins with 5% skin milk at room temperature for 2 hours, the membranes were incubated with the primary antibodies specific to iNOS (1:1,000) and β-actin (1:1,000) at 4°C overnight and then treated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 hour. After being treated with Western Bright™ ECL reagent, the protein bands on the membrane were measured by C300.

8. ELISA of inflammatory cytokines IL-6, TNF-α, and IL-1β

RAW264.7 cells were seeded into 48-well plates (1×10^5 cells/well) overnight and treated with NTB at concentrations of 1.0, 0.3, and 0.1 mg/mL, or positive control Bay11-7082 (20 μM) for 2 hours. Then the cells were treated with LPS at 500 ng/mL in a 37°C incubator for 24 hours. ELISA kits were used to examine the amount of IL-1β, IL-6 and TNF-α in the supernatants following the manufacturer's protocols. Finally, the absorbance was detected at 450 nm using an ELx808 within 30 minutes.

9. NF-κB reporter assay

RAW264.7 cells and 293T cells were seeded in a 96-well plate coated with poly-D-lysine and transfected with pNF-κB-SEAP reporter plasmid using HilyMax. After 4 hours, the cells were replaced with fresh DMEM overnight. And the cells were treated with PMA at 30 ng/mL for 24 hours after being incubated with NTB at concentrations of 1.0, 0.3, and 0.1 mg/mL for 2 hours. Bay11-7082 (20 μM) was a positive control. Then 10 μL supernatants were reacted with 100 μL QUANTI-Blue solution for 4 hours. The absorbance was detected at 630 nm using a ELx808.

10. Statistical analysis

Statistical data differences were assessed using GraphPad Prism version 5.01 (GraphPad Software, Inc., San Diego, CA, USA) and one-way one-way analysis of variance. The results were expressed as the mean±standard error

of the mean. All p-values less than 0.05 were considered statistically significant.

Results»»»»

1. Effect of NTB on cell viability

MTT assay indicating cytotoxicity was performed to detect safety of NTB to RAW264.7 cells and 293T cells. NTB at concentrations of 1.0, 0.3, and 0.1 mg/mL did not affect the growth of RAW264.7 cells and 293T cells (Figs. 1A and 1C). And the microscopic cell images also showed that NTB did not change the morphology of the RAW264.7 cells and 293T cells (Figs. 1B and 1D). These results suggest that NTB is not cytotoxic to RAW264.7 cells and 293T cells.

2. Effect of NTB on NO production and iNOS expression in LPS-stimulated cells

The anti-inflammatory effect of NTB was studied by using a Griess reagent to evaluate the level of NO in LPS-stimulated RAW264.7 cells. In RAW264.7 cells, the production of NO was significantly increased after the stimulation of LPS (500 ng/mL), which was inhibited by NTB treatment (Fig. 2A). In particular, compared with LPS treatment alone, NTB at a concentration of 1 mg/mL showed over 90% of the inhibitory effect (Fig. 2A). NO production is regulated by iNOS enzyme. Therefore, we also examined whether NTB affects iNOS protein expression by Western blot analysis. Before adding LPS (200 ng/mL), RAW264.7 cells were pretreated with NTB at concentrations of 1.0, 0.3, and 0.1 mg/mL, or Bay11-7082 (20 μM) for 2 hours. Western blot analysis confirmed that LPS increased the cumulation of iNOS protein in RAW264.7 cells (Fig. 2B). NTB showed a significant inhibitory effect on iNOS expression.

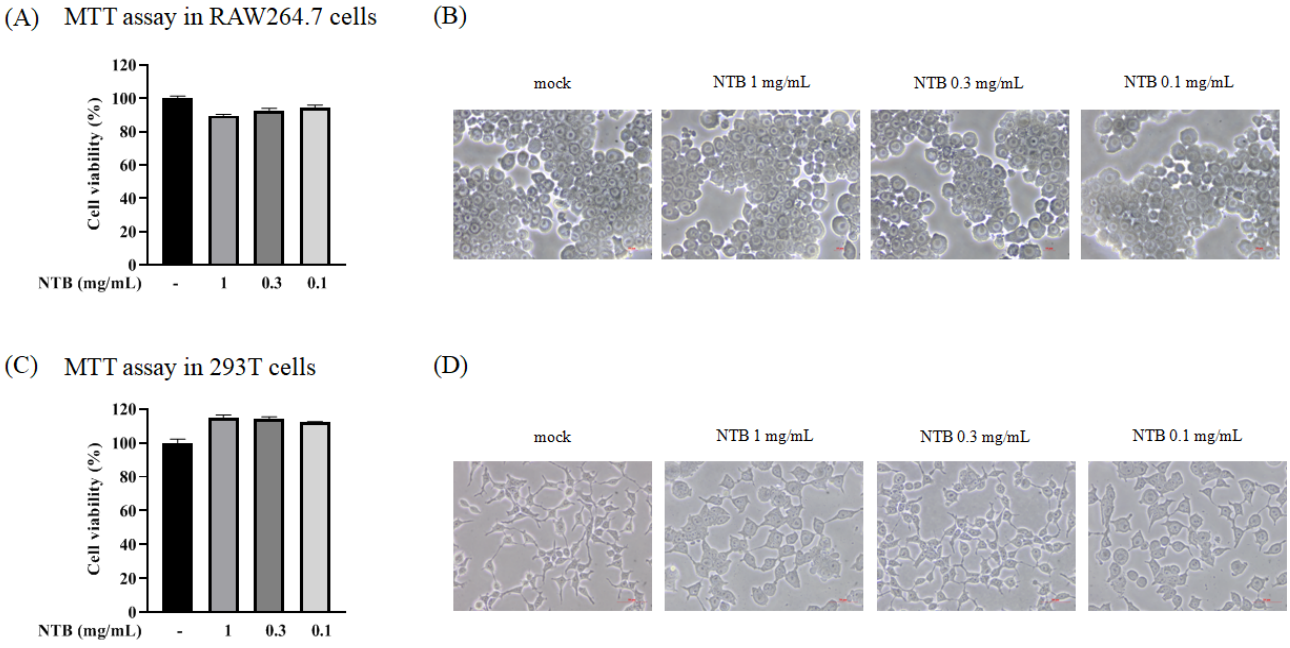

Fig. 1. Effect of NTB on cell viability. (A) RAW264.7 cells and (C) 293T cells were treated with NTB for 24 hours and the cell viability was tested by MTT assay. (B) RAW264.7 cell and (D) 293T cell images were taken under the microscope. Experimental data were presented as means±standard error of the mean. NTB: *Naetakbaekryeom-san*, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

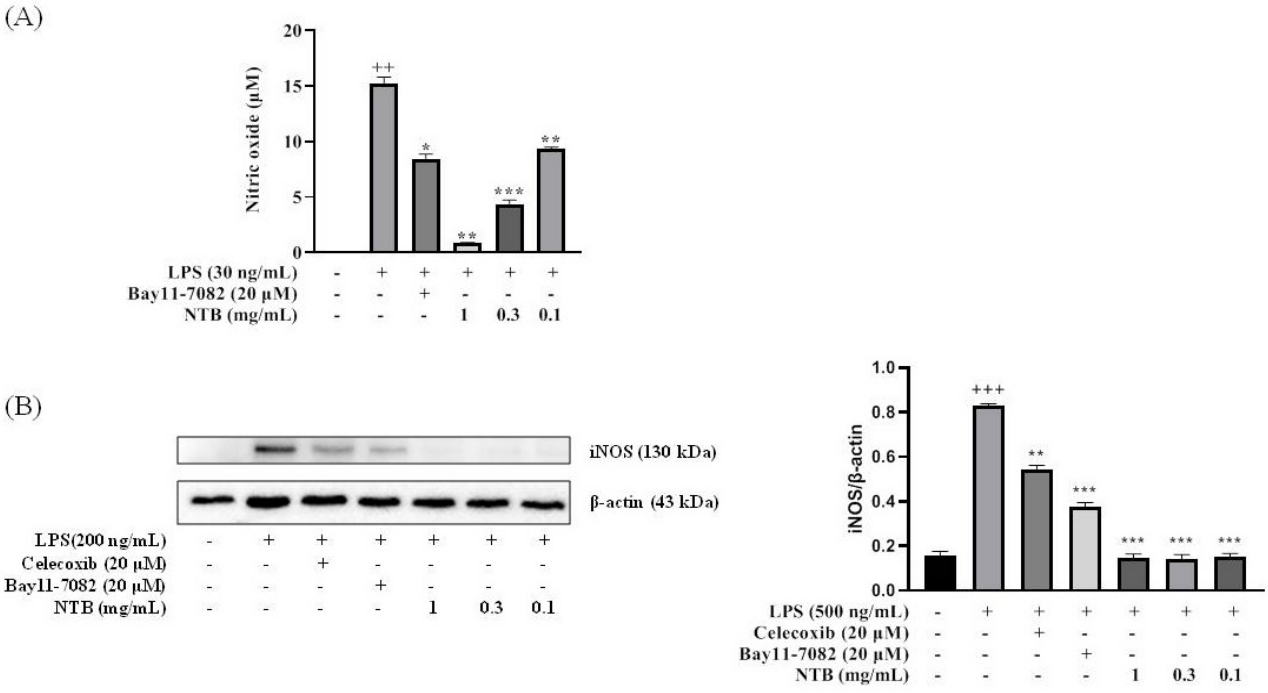

Fig. 2. Effect of NTB on NO production and iNOS expression in LPS-stimulated RAW264.7 cells. (A) RAW264.7 cells were treated with NTB for 2 hours and then stimulated with LPS (500 ng/mL) for 24 hours. The Griess reagent was used to measure the amount of NO secreted in the cell culture supernatant. (B) Effect of NTB on the expression of iNOS in LPS-stimulated RAW264.7 cells. RAW264.7 cells were pretreated with different concentrations of NTB or Bay11-7082 (20 μM) for 2 hours and then treated with LPS (200 ng/mL) for 24 hours. The protein expression of iNOS was confirmed by Western blot analysis. Bay11-7082 was used as a positive control. A house keeping protein β-actin was used as a control protein. The protein bands of iNOS were quantitatively evaluated by C300. Experimental data were presented as means±standard error of the mean ($^{++}p<0.01$ compared with the control group, $^{+++}p<0.001$ compared with the control group and *p<0.05, **p<0.01, ***p<0.001 compared with LPS groups). NTB: *Naetakbaekryeom-san*, NO: nitric oxide, iNOS: inducible NO synthase, LPS: lipopolysaccharides.

3. Effect of NTB on the production of inflammatory mediators in LPS-stimulated RAW264.7 Cells

In order to study the anti-inflammatory effect of NTB, we measured the amount of IL-1β, TNF-α, and IL-6 cytokines in LPS-stimulated RAW264.7 cells. The results showed that both positive control Bay11-7082 (20 μM) and NTB showed significant inhibitory effects on the production of IL-1β and IL-6 (Figs. 3A and 3B). Furthermore, NTB appeared to be more effective than Bay11-7082 (20 μM). TNF-α cytokine level was not regulated by NTB (Fig. 3C).

4. Effect of NTB on NF-κB activation in PMA-stimulated cells

A transcription factor NF-κB is a key regulator of inflammatory. NF-κB induces the expression of a variety of pro-inflammatory genes. Therefore, we examined the effect of NTB on the transcriptional activation of NF-κB. NTB inhibited the PMA-stimulated transcriptional activation of NF-κB in RAW264.7 cells (Fig. 4A) and 293T cells (Fig. 4B). These results indicated that NTB at a concentration of 1 mg/mL showed the most significant reduction in NF-κB transcriptional activation.

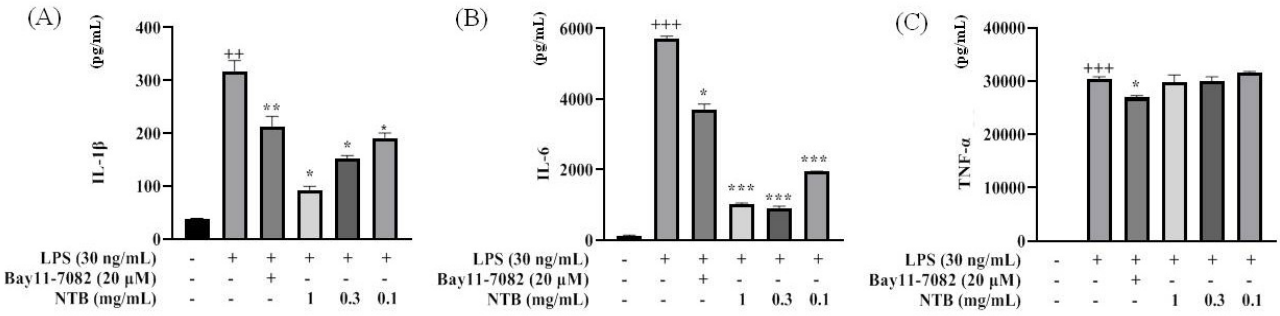

Fig. 3. Effect of NTB on the production of inflammatory mediators in LPS-stimulated RAW264.7 Cells. The RAW264.7 cells seeded into 48-well plates were treated with NTB (1.0, 0.3, 0.1 mg/mL) or Bay11-7082 (20 μM) for 2 hours, and then the cells were incubated with LPS (500 ng/mL) for 24 hours. IL-1β (A), IL-6 (B), and TNF-α (C) in the supernatants were measured by ELISA. Experimental data were presented as means±standard error of the mean ($^{++}$p<0.01 compared with the control group, $^{+++}$p<0.001 compared with the control group and *p<0.05, **p<0.01, ***p<0.001 compared with LPS groups). NTB: *Naetakbaekryeom-san*, LPS: lipopolysaccharides, IL: interleukin, TNF-α: tumor necrosis factor alpha, ELISA: enzyme-linked immunosorbent assay.

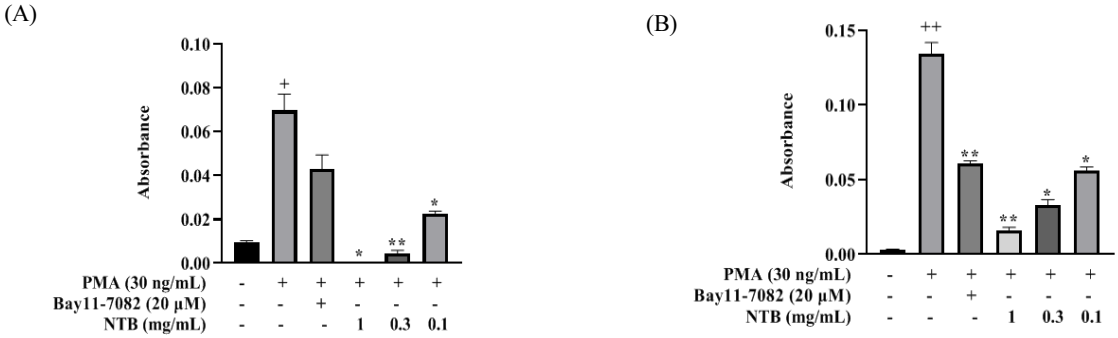

Fig. 4. Effect of NTB on NF-κB activation in PMA-stimulated cells. Both (A) RAW264.7 cells and (B) 293T cells seeded into poly-D-lysine hydrobromide-coated 96-well plates were transfected by pNF-κB-SEAP DNA using Hily Max. And then the cells were treated NTB with different concentrations or Bay11-7082 (20 μM) for 2 hours and stimulated with PMA (30 ng/mL) for 24 hours. SEAP activity was determined using the QUANTI-Blue assay system. Experimental data were presented as means±SEM from three independent experiments. ±SEM from three independent experiments. *p<0.05, **p<0.01. NTB: *Naetakbaekryeom-san*, NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells, PMA: phorbol 12-myristate 13-acetate, SEM: standard error of the mean. $^{+}$P<0.05 compared with the control group, $^{++}$P<0.01 compared with the control group.

Discussion and Counclusion»»»»

Inflammation is a reaction that attempts to regenerate and heal damage caused by physical stimuli, infections by microorganisms such as viruses, and bacteria, and toxins to tissues of living organisms. If inflammation is not controlled and continues for a long time, it can lead to excessive inflammatory reaction. Excessive inflammatory reaction is the causative mechanism of many diseases. It can lead to diseases, such a allergic reactions, autoimmune disease, atherosclerosis, ischemic heart disease, rheumatoid arthritis and neurodegenerative diseases like alzheimer. Therefore, proper control of inflammation is necessary[1,17].

NSAIDs are commonly used to suppress pathological inflammation, but side effects such as peptic ulcer, bleeding, and gastrointestinal mucosal damage have been reported[7,28]. In addition, steroids commonly used as anti-inflammatory drugs have side effects such as acne, increased risk of infection, osteoporosis, moon face, peptic ulcer, arteriosclerosis, weight gain and diabetes[8]. Research to reduce these side effects is in progress, and research on natural products for substitution and supplementation

is also being conducted[8,9].

NTB is a traditional Korean herbal medicine described in Man-Byeong-Hoi-Chun for the treatment of ruptured purulent axillary lumps that do not heal for a long time[10]. The anti-inflammatory efficacy of individual herbs in NTB *Paeoniae Radix*[12], *Angelicae Gigantis Radix*[13], *Forsythiae Fructus*[14], *Angelicae Dahuricae Radix*[15], *Ampelopsis Radix*[16], *Scutellariae Radix*[17], *Trichosanthis Fructus*[18], *Cnidii Rhizoma*[19], *Trichosanthis Radix*[20], *Olibanum*[21], *Bupleuri Radix*[22], *Saposhnikoviae Radix*[23], *Platycodi Radix*[24], *Tribuli Fructus*[25], and *Glycyrrhizae Radix*[26] has been reported, but the anti-inflammatory effects of NTB have not been studied, Therefore we studied the anti-inflammatory effects of NTB.

MTT assay indicating cytotoxicity performed to evaluate the safety of NTB in RAW264.7 and 293T cells (Fig. 1) confirmed that NTB is not cytotoxic to RAW264.7 and 293T cells.

NO is a highly reactive biogenerated radical that plays an important role in neurotransmission, vasodilation, and cell-mediated immunity. In general, NO is produced in large amounts by iNOS during the inflammatory response of macrophages stimulated by LPS. Under physiological conditions, NO plays an important role in signal transduction, but excessive NO formation causes inflammation tissue damage including nerve damage, and gene mutation. Therefore, inhibition of NO production is important for regulating the inflammatory response[27,29].

In this study, it was found that 1 mg/mL of NTB inhibits the production of NO by 90%, and 0.1, 0.3, 1 mg/mL of NTB significantly inhibits the expression of iNOS, which regulates the production of NO. These results suggest that NTB regulates inflammation by inhibiting iNOS expression and NO production.

LPS-stimulated macrophages increased the expression of inflammatory cytokines, such as IL-1β, IL-6 and TNF-α. IL-1β mediates inflammatory response and induces fever. It is also involved in T-cell activation, B-cell maturation, and NK cell activation, and acts on the hypothalamus to induce fever. Because IL-6 promotes protein synthesis in the acute phase, its level increases during infection and trauma, which promotes B-cell proliferation and differentiation and stimulates antibody secretion[27,30]. TNF-α not only mediates the inflammatory response by activating endothelial cells and neutrophils, but also induces the expression of adhesion molecules on the surface of vascular endothelial cells. NTB has been shown to be more effective in inhibiting the production of IL-1β and IL-6 than the positive control Bay11-7082 (20 μM), and TNF-α levels were not regulated by NTB. TNF-α is involved in the pathological process of autoimmune inflammatory diseases such as rheumatoid arthritis and ulcerative bowel disease[30]. Therefore, NTB is considered to be more effective in general inflammatory diseases, not autoimmune inflammatory diseases that require TNF-α inhibition.

NF-κB is a major mechanism for regulation of inflammatory cytokine production which is induced by LPS. NF-κB which is activated by the phosphorylation and degradation of *IκB-α* causes the translocation of NF-κB-p65 to the nucleus. This, in turn, activates specific genes transcription, such as those coding IL-6 and TNF-α. NF-κB also induces iNOS and cyclooxygenase-2[27,31]. NTB indicates that it inhibits inflammatory responses by reducing PMA-stimulated transcriptional activation of NF-κB.

In this study, NTB is evaluated to be significant in that it has an anti-inflammatory effect and provides a basis for application to the treatment of various general inflammatory diseases through an inflammatory suppression mechanism.

However further studies such as comparison of anti-inflammatory effects and antioxidant effects by NTB component, comparison of effects according to extraction solvents, and clinical studies are needed.

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