동의신경정신과 학회지
J. of Oriental Neuropsychiatry
Vol. 19. No. 2, 2008

Effect of Chrysanthemum zawadskii var. latilobum on the release of inflammatory mediators from LPS-stimulated mouse macrophages

Jung-Ho Lee, Ee-Hwa Kim*, Jae-Hyok Lee.
Dept. of Oriental Neuropsychiatry, College of Oriental Medicine, Semyung University, Dept. of AM-Meridian & Pointology, College of Oriental Medicine, Semyung University*

九折草가 LPS로 炎症誘導된 大食細胞柱에 미치는 影響

이정호, 김이화*, 이재혁
세명대학교 한의과대학 한방신경정신과학교실, 세명대학교 한의과대학 경혈학교실*

요약

목적 : 구절초는 국화과에 속하는 다년생 초본으로 가을에 줄기와 잎을 가을에 채취한 것을 한약재로 사용하여 대한, 월경불순 등 각종 여성질환과 함께 위냉증, 소화불량, 감고, 폐렴, 기관지염, 배뇨장애 및 신경퇴행성 질환에 활용되고 있다. 본 연구에서는 LPS로 염증유도된 대식세포주를 활용하여 구절초의 항염증효능을 확인하고자 하였다.

방법 : 구절초 추출물의 항염증효과를 관찰하기 위하여 RAW264.7 대식세포주에서 MTT cytotoxic assay, iNOS 및 COX-2 발현, NO와 PGE2 생성 및 NF-κB 활성실험을 수행하였다.

결과 : 세포독성실험을 통하여 구절초 추출물의 안전성이 확인되었으며 LPS로 염증유도된 RAW264.7 대식세포주에서 증가된 iNOS의 발현이 감소되었음을 확인하였다. 또한 NO 및 PGE2의 생성을 억제하였다. 이러한 결과는 구절초가 염증매개물질의 생성을 억제하는 효과가 있음을 확인할 수 있었으며, NF-κB 활성도를 감소시킴을 관찰하였다.

결론 : 이러한 결과는 구절초가 NF-κB pathway를 조절해 줌으로써 항염증효과를 나타내는 것으로 사료된다.

주제어 : 구절초, 대식세포, 항염증, LPS, NF-κB

투고일 : 6/5 수정일 : 7/6 채택일 : 7/14
* 교신저자 : 이정호 주소 : 서울 강북구 수유3동 175-52 양지한의원
Tel : 02-907-8002 E-mail : lj1217@paran.com

I. INTRODUCTION

Macrophages are a type of differentiated tissue cells that originate blood monocyte. The cells have several functions such as: (1) the removal of cell debris: (2) killing the pathogenic microorganisms ; (3) the processing and presentation of antigens ingested to lymphocytes[1]. Therefore, the activation of macrophages is a key event for effective innate and adaptive immunity. When the body is stimulated by pathologic stimuli or injury, macrophages release: (1) numerous pro-inflammatory cytokine [e.g. tumor necrosis factor (TNF)-α, and interleukin (IL)-1β]; (2) chemokines and chemoattractants [e.g. IL-8 and monocyte chemoattractant protein (MCP)-1]; (3) cytotoxic and inflammatory molecules [e.g. nitric oxide (NO), reactive oxygen species (ROS) and prostaglandin E_2 (PGE_2)]. In addition, the macrophages up-regulate the surface levels of glycoproteins (e.g. costimulatory molecules [CD80 and CD86] and adhesion molecules [e.g. selectins and integrins])[1-3].

Chronic (or acute) inflammation is a multiple process, which is mediated by activated inflammatory or immune cells, From them, macrophages play a central role in managing many different immunopathological phenomena such as the over - production of pro-inflammatory cytokines and inflammatory mediators [reactive oxygen species (ROS), nitric oxide (NO) and PGE_2], generated by activated inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX) - 2[4,5]. Under inflammatory conditions, immune cells are also stimulated by adhesion molecule activations signals in order to enhance the migration capacity to inflamed tissue[4-6] and finally to form heterotypic cell clustering (adhesion) between the immune cells, endothelial cells and inflamed cells[7]. Indeed, a number of inflammatory stimuli such as lipopolysaccharide (LPS) and pro-inflammatory cytokines activate immune cells to up - regulate such inflammatory states[8] and therefore, these are useful targets for developing new anti - inflammatory drugs and exploring the molecular anti-inflammatory mechanisms of a potential drug.

The extract from *Chrysanthemum zawadskii var. latilobum* (CZ), commonly known in Korea as Gu-Jul-Cho, is used in traditional medicine to treat pneumonia, bronchitis, cough, common cold, pharyngitis, bladder-related disorders, women's diseases, gastroenteric disorders, and hypertension[9].

In this study, we evaluated the potential of CZ as a therapeutic modality for inflammation in RAW264.7 mouse macrophages cells. Our results showed that CZ extract largely inhibited the excessive production of inflammatory mediators such as NO and PGE_2 through the down-regulation of their transcription factors and the modulation of the NF-κB-dependent pathway.

II. MATERIALS AND 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 cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL) at 37℃ in 5% CO_2-95% O_2 in a humidified cell incubator.

2. Preparation of extract

Chrysanthemum zawadskii var. latilobum (CZ) was purchased from Kyung-Dong herbal market (Seoul, Korea), and the identification of plant material was done by Dr. Kang-Hyun Leem (Professor, Department of Herborogy, College of Oriental Medicine, Semyung University). The voucher specimen was deposited at the college of Oriental medicine, Semyung University. To obtain the water extract of CZ, 200 g of CZ was added to distilled water, and extraction was performed by heating at 80 ℃, concentrated with a rotary evaporator and lyophilized. The resulting powder, weighing 30 g, was dissolved in saline.

3. MTT cytotoxicity assay

Cell viability was determined using the MTT assay kit (Boehringer Mannheim GmbH, Mannheim, Germany) as per the manufacturer's protocols. In order to determine the cytotoxicity of CZ, cells were treated with CZ at concentrations of 5 ㎍/㎖, 10 ㎍/㎖, 25 ㎍/㎖, 50 ㎍/㎖, 100 ㎍/㎖ and 250 ㎍/㎖ for 24 h. Cultures of the control group were left untreated. 10 ㎖ of the MTT labeling reagent was added to each well, and the plates were incubated for 4 h. Solubilization solution of 100 ㎖ was then added to each well, and the cells were incubated for another 12 h. The absorbance was then measured with a microtiter plate reader (Bio-Tek, Winooski, VT, USA) at a test wavelength of 595 ㎚ and a reference wavelength of 690 ㎚. Optical density (O.D.) was calculated as the difference between the absorbance at the reference wavelength and that at the test wavelength. Percent viability was calculated as (O.D. of drug-treated sample/control O.D.) x 100.

4. RNA isolation and RT-PCR

RAW264.7 cells (1 x 10^5 cells/mL) were pretreated with 50 and 100 ㎍/㎖ of CZ extract for 30 min, and were then stimulated with 1 ㎍/㎖ of LPS or without LPS for 2 h. Total RNA from each cell was prepared by adding TRIzol reagent according to the protocol of the manufacturer (GibcoBRL). The total RNA solution was stored at -70 ℃ until use.

To identify expressions of iNOS and COX-2 mRNAs, RT-PCR was performed. Total RNA was isolated from RAW 264.7 cells using RNAzol^{TMB} (TEL-TEST,

Friendswood, TX, USA). Two μg of RNA and 2 μℓ of random hexamers (Promega, Madison, WI, USA) were added together, and the mixture was heated at 65℃ for 10 min. One μℓ of AMV reverse transcriptase (Promega), 5 μℓ of 10 mM dNTP (Promega), 1 μℓ of RNasin (Promega), and 5 μℓ of 10 x AMV RT buffer (Promega) were then added to the mixture, and the final volume was brought up to 50 μℓ with diethyl pyrocarbonate (DEPC)-treated water. The reaction mixture was then incubated at 42 ℃ for 1 h.

PCR amplification was performed in a reaction volume of 40 μℓ containing 1 μℓ of the appropriate cDNA, 1 μℓ of each set of primers at a concentration of 10 pM, 4 μℓ of 10 x RT buffer, 1 μℓ of 2.5 mM dNTP, and 2 units of Taq DNA polymerase (TaKaRa, Shiga, Japan). For mouse iNOS, the primer sequences were 5' - GTGTTCCACCAGGAGATGTTG - 3' (a 21 - mer sense oligonucleotide) and 5' - CTCCTGCCCACTGAGTTCGTC-3' (a 21-mer anti-sense oligonucleotide). For mouse COX-2, the primer sequences were 5'-TGCATGTGGCTGTGGATGTCATCA A-3' (a 25-mer sense oligonucleotide) and 5'-CACTAAGACAG ACCCGTCATCTCCA -3' (a 25-mer anti-sense oligonucleotide). For cyclophilin, the internal control used in the study, the primer sequences were 5' - ACCCCACCGTGTTCTTCGAC-3' (a 20 - mer sense oligonucleotide starting at position 52) and 5' - CATTTGCCATGGACAAGATG - 3' (a 20 - mer anti - sense oligonucleotide starting at position 332). The expected sizes of the PCR products were 583 bp (for COX-2), 500 bp (for iNOS) and 299 bp (for cyclophilin).

For iNOS and COX-2, the PCR procedure was carried out using a GeneAmp 9600 PCR system (Perkin Elmer, Norwalk, CT, USA) under the following conditions: initial denaturation at 94 °C for 5 min, followed by 40 amplification cycles, each consisting of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec, and extension at 72 °C for 30 sec, with an additional extension step at the end of the procedure at 72 °C for 5 min. For cyclophilin, the PCR procedure was carried out under identical conditions except that 25 amplification cycles were executed. The final amount of RT-PCR product for each of the mRNA species was calculated densitometrically using Molecular Analyst™ version 1.4.1 (Bio-Rad, Hercules, CA, USA).

5. Western blot analysis

RAW264.7 cells (1 x 10^5 cells/mℓ) were pretreated with 50 and 100 μg/mℓ of CZ extract for 30 min and stimulated with 1 μg/mℓ of LPS or without LPS for 24 h. Cells were lysed with 0.1 mℓ of 50 mM Tris-HCl, pH 7.2, containing 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.15 M NaCl and 1% NP-40. The cell lysates were assayed for protein concentration by the Bradford staining method, and equal amounts of protein (20 μg/mℓ) were then electrophoresed on 8-10% density SDS-acrylamide gels. After electrophoresis, the proteins were transferred from the gel to a nitrocellulose (NC) membrane using an electric transfer

system. Non-specific binding was blocked with 3% skim milk in TBS-T buffer (5 mM Tris-HCl, pH 7.6, 136 mM NaCl, 0.1 % Tween-20) for 1 h. Blots were incubated at room temperature for 1 h each with primary anti-body against iNOS (1:3000 dilutions; Santa Cruz Biothchnology, Santa Cruz, CA), actin (1:8000; Sigma) added, and were then washed three times with 1x TBS-T. Blots were incubated for 1 h at room temperature (RT) with a 1:10,000 dilutions of horseradish peroxidase (HRP)-labeled anti-mouse IgG (1:1000, Santa Cruz Biotechnology), and were then washed three times with 1x TBS-T. Blots were developed with the ECL Western detection reagents (Amersham Bioscience, Piscataway, NJ).

6. DNA binding assay of nuclear factor-κB (NF-κB)

RAW264.7 cells (1 x 10^5 cells/㎖) were pretreated with 100 ㎍/㎖ of CZ extract for 30 min and stimulated with 1 ㎍/㎖ of LPS or without LPS for 30 min. Nuclear extracts were prepared by NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Inc., Rockford, IL) according to the protocol of the manufacturer. In brief, the cells in six wells were washed with ice-cold PBS. The cells were then scraped, transferred to microtubes, and allowed to swell after the addition of cytoplasmic extraction reagent (CER). The lysates were incubated for 10 min on ice and centrifuged at 12,000 rpm for 5 min at 4 ℃. Pellets containing crude nuclei were resuspended in nuclear extraction reagent (NER) solution and incubated in ice for 1 h. The samples were centrifuged at 12,000 rpm for 10 min to obtain nuclear fractions. The nuclear fractions wer assayed for protein concentration by using the Bradford staining method, and equal amounts of protein were then electrophoresed on 10% SDS - acrylamide gels. After electrophoresis, the proteins were transferred frome the gel to an NC membrane using an electric transfer system. Non-specific binding was blocked with 3# skim milk in TBS-T buffer (5 mM Tris-HCl, pH 7.6, 136mM NaCl, 0.1% Tween-20) for 1 h. Blots were incubated at room temperature for 1 h each with primary antibody against p65 NF-κB (1:5000 dilution; Santa Cruz Biotechnology) and actin (1:8000; Sigma), and were then washed three times with 1x TBS-T. Blots were incubated for 1 h at room temperature with a forseradish peroxidase (HRP)-labeled anti-rabbit IgG (1:1000; Santa Cruz Biotechnology), and were then washed three times with 1x TBS-T. Blots were developed with ECL Western detection reagents (Amersham Bioscience)

7. Measurement of nitric oxide production

In order to determine the effect of CZ on NO production, the amount of nitrite in the supernatant was measured using a commercially available NO detection kit (Intron Biotech., Seoul, Korea). After collection of 100 ㎖ of supernatant, 50 ㎖ of N1 buffer was added to each well, and the plate was incubated at room temperature for

10 min. N2 buffer was then added, and the plate was incubated at room temperature for 10 min. The absorbance of the content of each well was measured at 450 ㎚. The nitrite concentration was calculated from a nitrite standard curve.

8. Measurement of prostaglandin E2

Synthesis Assessment of PGE_2 synthesis was performed using a commercially available PGE_2 competitive enzyme immunoassay kit (Amersham Pharmacia Biotech. Inc., Piscataway, NJ, USA). Cells were lysed and cell lysates and standards were put into different wells on the goat anti-mouse IgG-coated microtiter plate provided in the kit. Mouse anti-PGE_2 antibody and peroxidase-conjugated PGE_2 were added to each well, and the plate was incubated at room temperature and shook for 1 h. The wells were drained and washed, and 3,3',5,5'-tetramethylbenzidine/hydrogen peroxide solution was added. The plate was incubated at room temperature with shaking, and the reaction was stopped after 30 min through the addition of H_2SO_4. The absorbance of the content of each well was then measured at 450 ㎚.

9. Statistical analysis

Results are expressed as mean ± standard error mean (S.E.M.). Data were analyzed by student's *t*-test using SPSS (Ver. 10.0). Differences were considered statistically significant at $p<0.05$.

III. RESULTS

1. Cell toxicity of CZ extract

The viabilities of cells incubated with *Chrysanthemum zawadskii var. latilobum* (CZ) at 5 ㎍/㎖, 10 ㎍/㎖, 25 ㎍/㎖, 50 ㎍/㎖, 100 ㎍/㎖ and 250 ㎍/㎖ for 24 h were 82.95 ± 12.50, 89.20 ± 0.85%, 89.77 ± 3.12%, 90.05 ± 0.85%, 91.69 ± 1.27 and 93.75 ± 1.98% of the control value, respectively. The MTT assay revealed that CZ exerted no significant cytotoxicity in the macrophage RAW 264.7 cells (Fig. 1).

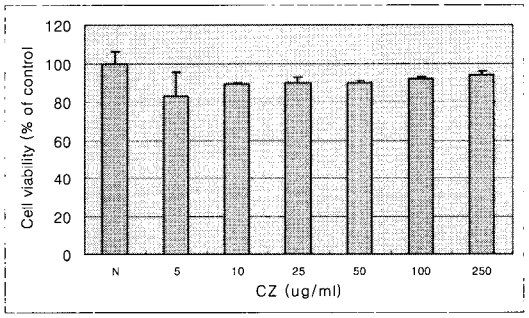

Fig. 1. Cytotoxic effect of Chrysanthemum zawadskii var. latilobum (CZ) extract in RAW264.7 cells were determined via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Three independent experiments were performed, and data shown represent the mean ± S.E.M.

2. iNOS and COX-2 expression in macrophages

To ensure that CZ extract-mediated inhibition of LPS-induced NO production resulted from the inhibition of iNOS and COX-2 activity and not to its expression, RT-PCR and Western blot analysis of iNOS and COX-2 mRNA and protein were performed. In the present study, the mRNA

levels of iNOS and COX-2 in the control cells were used as a control value 1.00. The levels of mRNAs were markedly increased by treatment with 1 μg/mℓ LPS, while significantly decreased in the levels of iNOS mRNAs treated with CZ at 50 μg/mℓ and 100 μg/mℓ (Fig. 2).

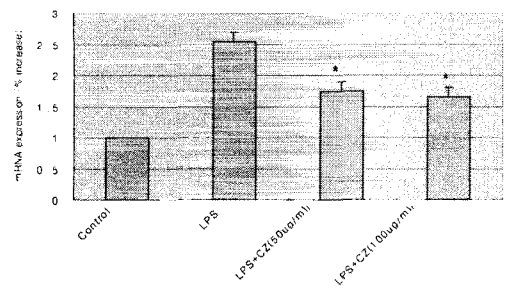

Fig. 2. Effect of Chrysanthemum zawadskii var. latilobum (CZ) extract on LPS-induced mRNA of iNOS in macrophages. RAW264.7 cells were incubated with CZ extract in the absence or presence of LPS (1 μg/mℓ). The mRNA levels of iNOS was determined by RT-PCR. As the internal control, cyclophilin mRNA was also reverse-transcribed and amplified. *represents $p<0.05$ compared to the lipopolysaccharide (LPS)-treated group.

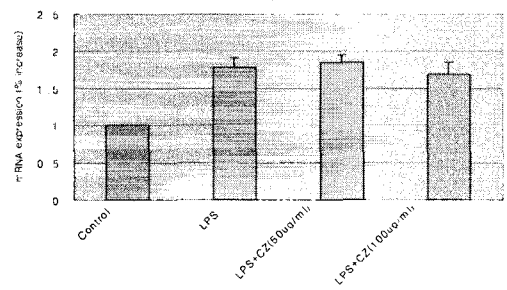

Fig. 3. Effect of Chrysanthemum zawadskii var. latilobum (CZ) extract on LPS-induced mRNA of COX-2 in macrophages. RAW264.7 cells were incubated with CZ extract in the absence or presence of LPS (1 μg/mℓ). The mRNA levels of COX-2 was determined by RT-PCR. As the internal control, cyclophilin mRNA was also reverse-transcribed and amplified.

3. DNA binding assay of nuclear factor-κB

LPS-induced phosphatidylinositol 3-kinase (PI3-kinase)/Ark and MAPKs can activate a variety of transcription factors, including NF-κB, which coordinates the induction of many inflammatory cytokines. We investigated whether the CZ extract modulates the inflammation-mediated signal pathway in RAW264.7 cells using Western blot analysis. The CZ extract attenuated the LPS-elicited nuclear translocation of NF-κB protein in RAW264.7 cells (Fig. 4). These findings suggest that the CZ extract was associated with down-regulation or degradation of NF-κB p65 protein in LPS-stimulated macrophages.

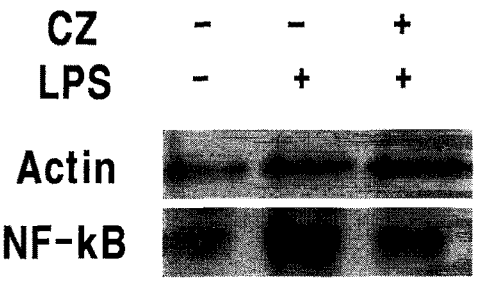

Fig. 4. Effect of Chrysanthemum zawadskii var. latilobum (CZ) extract on LPS-induced nuclear translocation of NF-κB in RAW264.7 cells. The CZ extract attenuated the LPS-elicited nuclear translocation of NF-κB protein in RAW264.7 cells. These findings suggest that the CZ extract was associated with down-regulation or degradation of NF-κB p65 protein in LPS-stimulated macrophages.

4. NO systhesis assay

Because NO is known to be a proinflammatory mediator in inflammatory diseases, we investigated whether the CZ extract inhibits NO production from the macrophages that were activated with LPS.

From NO detection assay, after 24 h of exposure to LPS, the amount of nitrite was increased from 7.69 ± 0.19 μM to 35.63 ± 0.04 μM, while decreased to 23.44 ± 0.24 μM, 23.35 ± 0.28

μM and 26.64 ± 0.55 μM by treatment with CZ at 50 ㎍/㎖, 100 ㎍/㎖ and 250 ㎍/㎖ (Fig. 5).

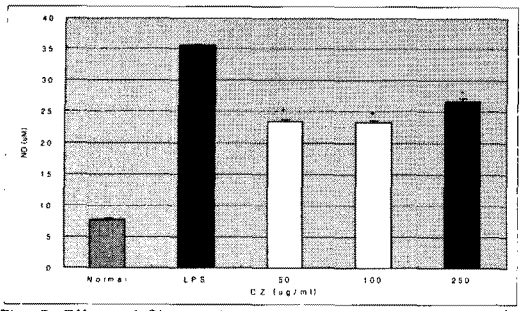

Fig. 5. Effect of Chrysanthemum zawadskii var. latilobum (CZ) extract on LPS-induced nitric oxide (NO) production in macrophages. RAW264.7 cells were incubated with different concentrations of CZ extract in the presence of LPS (1 ㎍/㎖) for 24 h. NO was measured by the Griess reaction. Three independent experiments were performed, and data represent are the mean ± S.E.M. *represents $p<0.05$ compared to the lipopolysaccharide (LPS)-treated control group.

5. PGE2 synthesis assay

From PGE_2 immunoassay, after 24 h of exposure to LPS, the amount of PGE_2 was increased from 5.97 ± 1.28 pg/well to 45.37 ± 5.42 pg/well, while decreased to 33.85 ± 4.84 pg/well and 30.24 ± 4.43 pg/well by the treatment with CZ at 100 ㎍/㎖l and 250 ㎍/㎖ (Fig. 6).

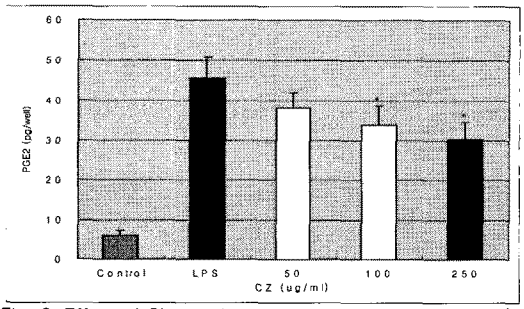

Fig. 6. Effect of Chrysanthemum zawadskii var. latilobum (CZ) extract on LPS-induced prostaglandin E2 (PGE2) production in macrophages. RAW264.7 cells were incubated with different concentrations of CZ extract in the presence of LPS (1 ㎍/㎖) for 24 h. PGE2 was measured by the Griess reaction. Three independent experiments were performed, and data represent are the mean ± S.E.M. *represents $p<0.05$ compared to the lipopolysaccharide (LPS)-treated group.

IV. DISCUSSION and CONCLUSION

Chrysanthemum zawadskii var. latilobum (CZ) has been used in traditional medicine in Korea for the treatment of pneumonia, bronchitis, cough, common cold, pharyngitis, bladder-related disorders, gastroenteric disorders and hypertension[9]. Linarin is the main active compound found in the herb CZ. It is an acacetin-7-β-rhamnoglucoside, called aca-ciin. Linarin has significant beneficial therapeutic effects[10]. Despite the identified effects in several diseases, the pharmacological activities of the CZ extract have not been fully explored with regard to its immunological effects. Therefore, in this study, we prepared CZ extract for treatment of the patients with inflammatory diseases, including chronic renal failure, rheumatoid arthritis, and neurodegenerative diseases, and then identified its anti-inflammatory effects in LPS-activated mouse macrophages.

In mammalian cells, NO is synthesized by three different isoforms of NOS, namely, nNOS, eNOS and iNOS. Although nNOS and eNOS are constitutively expressed, iNOS is expressed in response to interferon-γ, LPS and to a variety of proinflammatory cytokines[11,12]. COX in the enzyme that converts arachidonic acid to PGs. Like NOS, COX existed in two isoforms, i.e., COX-1 and COX-2[13]. COX-1 is expressed constitutively in most tissues and appears to be reponsible for maintaining normal physiological

functions. In contrast, COX-2 is detectable in only certain types of tissues and is induced transiently by growth factors, proinflammatory cytokines, tumor promoters and bacterial toxins[14,15]. Moreover, elevated levels of COX have been detected in different tumor types and this may account for the excessive production of inflammatory PGs[16].

NO is produced by activated macrophages as a result of induction by several stimuli, including TNF-α, IFN-γ and LPS, and may contribute to the pathological process in various acute and chronic inflammatory conditions[17-19]. Therefore, the reduction of NO production, including inhibition of iNOS gene activity, depletion of arginine substrate by arginase, and transcriptional down-regulation of iNOS gene expression by endogenous or exogenous manipulation, may present a useful strategy for the treatment of a variety of inflammatory diseases, including some neurological disorders[20 22]. In this study, we demonstrate that CZ extract significantly inhibited NO production (Fig. 5) in LPS-stimulated macrophages without showing any cell toxicity (Fig. 1). Furthermore, the inhibitory action of CZ extract on LPS-induced NO production appears to involve the inhibition of iNOS gene expression (Fig. 2).

LPS stimulation of macrophages activates several extracellular signaling pathways, including the IκB kinase (IKK)-NF-κB pathway[23]. Proinflammatory cytokines that are released from macrophages are also involved in many aspects of inflammatory events, including expression of iNOS and COX-2 involved the mitogen-activated protein kinase 3 (MKK3)/p38 MAPK and NF-κB pathways[24,25]. These signaling pathways activated the induction of many genes that encode inflammatory mediators. Therefore, these signaling molecules may represent novel targets for the treatment of patients with inflammatory diseases[26].

NF-κB is one of the most ubiquitous transcription factors and regulates gene involved in cellular proliferation, inflammatory responses and cell adhesion. The activation of NF-κB has been reported to induce the transcriptions of many pro-inflammatory mediators, e.g., iNOS, COX-2, TNF-α and interleukin-1β, -6 and -8[27,28].

NF-kB is known to play a critical role in the regulation of cell survival genes and to coordinate the expressions of proinflammatory enzymes and cytokines, such as iNOS, and COX-2[29 33]. Since the expressions of these pro-inflammatory mediators are known to be modulated by NF-κB. NF-κB is essentially composed of two proteins, p50 and p65, which are also referred to as RelA and cRel, respectively[34]. In the resting state, NF-κB is bound in the cytosol with an inhibitory protein, I-κB. However, following its induction by a variety of agents such as LPS, TNF-α and tissue plasminogen activator, I-κB is phosphorylated and this process triggers its proteolytic degradation via 26s proteosome, which releases NFκB from I-κB and allows its translocated to the nucleus, where it binds to NF-κB binding sites in the promoter regions of its target

genes. In the present study, we found that CZ blocked the LPS-induced activation of NF-κB by inhibiting its translocation to the nucleus[35].

PGE_2, a major metabolite of the COX-2 pathway, has emerged as an important lipid mediator of inflammatory and immunoregulatory processes. PGE_2 is implicated in the pathogenesis of acute and chronic inflammatory states[36], and specific COX-2 inhibitors attenuate the symptoms of inflammation[37]. COX activity and subsequent production of PGE_2 are closely related to the generation of NO radicals[38]. Salvemini et al.[39] reported that NO modulates the activity of COX-2 as cGMP-independently and plays a critical role in the release of PGE_2 by direct activation of COX-2. Inhibition of the iNOS expression in murine macrophages can be suggested as another possible mechanism of non-steroidal anti-inflammatory drugs[40].

In the present study, we examed the effects of CZ on LPS-induced pro-inflammatory molecules, including NO and PGE_2. CZ was found to be the effective inhibitor of these mediators. To further explore the possible mechanism of these inhibitions by CZ, we investigated the effects of CZ extract on LPS-induced activation of the NF-κB pathways. We showed that the anti-inflammatory effect of CZ extract was associated with decreases in LPS-induced nuclear translocation of NF-κB in activated macrophages. These results indicate the potent activity of CZ extract as a modulator of LPS-mediated NF-κB pathway in activated macrophages.

In conclugion, we demonstrate here that CZ extract is a potent suppressor of secretion of an inflammatory mediator, NO, PGE_2 and proinflammatory cytokines in LPS-activated macrophages. Thus, CZ extract may have therapeutic potential for the modulation and regulation of macrophage activation, and may provide safe and effective treatment options for a variety of inflammation-mediated diseases.

Reference

1. Kinne RW, Brauer R. Stuhlmuller B, Palombo-Kinne E, Burmester GR. Macrophages in rheumatoid arthritis. Arthritis Research. 2000;2:189-202.
2. Bresnihan B. Pathogenesis of joint damage in rheumatoid arthritis. Journal of Rheumatology. 1999;26:717-719.
3. Gracie JA, Forsey RJ, Chan WL, Gilmour A, Leung BP, Greer MR, Kennedy K, Carter R. Wei XQ, Xu D, Field M, Foulis A, Liew FY, McInnes IB. A proinflammatory role for IL-8 in rheumatoid arthritis. Journal of Clinical Investigation. 1999;104:1393-1401.
4. Lundberg IE. The role of cytokines, chemokines, and adhesion molecules in the pathogenesis of idiopathic inflammatory myopathies. Current Rheumatology Report. 2000;2:216-224.
5. Walsh LJ. Mast cells and oral inflammation. Critical Reviews in Oral Biology and Medicine. 2003;14:188-198.

6. Rochon YP, Frojmovie MM. A model for the recruitment of neutrophils as sites of inflammation. Physiological relevance of in vivo neutrophil aggregation. Medical Hypothesis. 1992;38:132-138.
7. Lowe JB. Glycan-dependent leukocyte adhesion and recruitment in inflammation. Current Opinion in Cell Biology. 2003;15:531-538.
8. Gallucci S, Provenzano C, Mazzarelli P, Scuderi F, Bartoccioni E. Myoblasts produce IL-6 in response to inflammatory stimuli. International Immunology. 1998;10:267-273.
9. 이창복. 대한식물도감. 서울:향문사. 1982:754.
10. Han S, Sung KH, Yim D, Lee S, Lee CK, Ha NJ, Kim K. The effect of Linarin on LPS-induced cytokine production and nitric oxide inhibition in murine macrophages cell line RAW264.7. Arch Pharm Res. 2002;25(2):170-177.
11. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacological Reviews. 1991;43(2):109-42.
12. Yun HY, Dawson VL, Dawson TM. Neurobiology of nitric oxide. Critical Reviews in Neurobiology. 1996;10:291-316.
13. Funk CD, Funk LB, Kennedy ME, Pong AS, Fitzgerald GA. Human platelet/erythroleukemia cell prostaglandin G/H synthase: cDNA cloning, expression, and gene chromosomal assignment. FASEB J. 1991;5(9):2304-12.
14. Hinz B, Brune K. Cyclooxygenase-2--10 years later. Journal of Pharmacology and Experimental Therapeutics. 2002;300(2):367-75.
15. Prescott SM, Fitzpatrick FA. Cyclooxygenase-2 and carcinogenesis. Biochimica et Biophysica Acta. 2000;1470(2);M69-78.
16. Subbaramaiah K, Dannenberg AJ. Cyclooxygenase 2: a molecular target for cancer prevention and treatment. Trends in Pharmacological Sciences. 2003;24(2):96-102.
17. Kilbourn RG, Belloni P. Endothelial cell production of nitric oxide in response to interferon-ɣ in combination with tumor necrosis factor, interleukin-1, or endotoxin. Journal of National Cancer Institute. 1990;82:772-776.
18. Nathan C. Nitric oxide as a secretory product of mammalian cells. The FASEB Journal. 1992;6:3051-3064.
19. Murakami A, Takahashi D, Hagihara K, Koshimizu K, Ohigashi H. Combinatorial effects of nonsteroidal anti-inflammatory drugs and food constituents on production of prostaglandin E2 and tumor necrosis factor-alpha in RAW264.7 murine macrophages. Bioscience Biotechnology and Biochemistry 2003;67:1056-1062.
20. Albina JL, Reichner JS. Role of nitric oxide mediation of macrophage cytotoxicity and apoptosis. Cancer Metastasis Review. 1998;17:39-53
21. Boucher JL, Moali C, Tenu JP. Nitric oxide biosynthesis, nitric oxide synthase inhibitors and arginase competition for L-arginine utilization. Cee Molecular Life

Science. 1999;55:1015-1028.

22. Lee HJ, Hyun EA, Yoon WJ, Kim BH, Rhee MH, Kang HK, Cho JY, Yoo ES. In vitro anti-inflammatory and anti-oxidative effects of Cinnamomum camphora extracts. Journal of Ethnopharmacology. 2006;103:208-216.
23. Kim SJ, Jeong HJ, MOon PD, Lee KM, Lee HB, Jung HJ, JUng SK, Rhee HK, Yang DC, Hong SH, Kim HM. Anti-inflammatory activity of gumiganghwaltang through the inhibition of nuclear factor-kappa B activation in peritoneal macrophages. Bilogical and Pharmaceutical Bulletin. 2005;28:233-237.
24. Wu MJ, Wang L, Ding HY, Weng CY, Yen JH. Glossogyne tenuifolia acts to inhibit inflammatory mediator production in a macrophage cell line by downregulating LPS-induced NF-kappa B. Journal of Biomedical Science. 2004;11:186-199.
25. Wang XM, Kim HP, Song R, Choi AM. Caveolin-1 confers anti-inflammatory effects in murine macrophages via the MKK3/p38 MAPK pathway. American Journal of Respiratory Cell and Molecular Biology. 2006;34:434-442.
26. Guha M, Mackman N. LPS induction of gene expression in human monocytes. Cellular Signal. 2001;13:85-94.
27. Lappas M, Permezel M, Georgiou HM, Rice GE. Nuclear factor kappa B regulation of proinflammatory cytokines in human gestational tissues in vitro. Biology of Reproduction. 2002;67(2):668-673.
28. Surh YJ, Chun KS, Cha HH, Han SS, Keum YS, Park KK, Lee SS. Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation. Mutation Research. 2001;480-481:243-68.
29. Chen F, Kuhn DC, Sun SC, Gaydos LJ, Demers LM. Dependence and reversal of nitric oxide production on NF-kappa B in silica and lipopolysaccharide-induced macrophages. Biochemical and Biophysical Research Communications. 1995;214(3):839-46.
30. Roshak AK, Jackson JR, McGough K, Chabot-Fletcher M, Mochan E, Marshall LA. Manipulation of distinct NFkappaB proteins alters interleukin-1beta-induced human rheumatoid synovial fibroblast prostaglandin E2 formation. Journal Biological Chemistry. 1996;271(49):31496-501.
31. Schmedtje JF, Ji YS, Liu WL, DuBois RN, Runge MS. Hypoxia induces cyclooxygenase-2 via the NF-kappaB p65 transcription factor in human vascular endothelial cells. Journal of Biological Chemistry. 1997;272(1):601-8.
32. Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. Journal of Biological Chemistry. 1994;269(7): 4705-8.
33. Xie W, Merrill JR, Bradshaw WS, Simmons DL. Structural determination and promoter analysis of the chicken mitogen-inducible prostaglandin G/H

synthase gene and genetic mapping of the murine homolog. Archives of Biochemistry and Biophysics. 1993;300(1):247-52.

34. Kracht M, Saklatvala J. Transcriptional and post-transcriptional control of gene expression in inflammation. Cytokine. 2002;20:91-106.

35. Delfino F, Walker WH. Hormonal regulation of the NF-kappaB signaling pathway. Molecular and Cellular Endocrinology. 1999;157(1-2):1-9.

36. Hinz B, Brune K, Pahl A. Prostaglandin E (2) upregulates cyclooxygenase - 2 expression in lipopolysaccharide - stimulated RAW 264.7 macrophages. Biochem Biophys Res Commun. 2000;272(3):744—8.

37. Yui Y, Hattori R, Kosuga K, Eizawa H, Hiki K, Kawai C. Purification of nitric oxide synthase from rat macrophages. J Biol Chem. 1991;266(19):12544—7.

38. Pang L, Hoult JR. Cytotoxicity to macrophages of tetrandrine, an antisilicosis alkaloid, accompanied by an overproduction of prostaglandins. Biochem Pharmacol. 1997;53(6):773—82.

39. Salvemini D, Misko TP, Masferrer JL, Seibert K, Currie MG, Needleman P. Nitric oxide activates cyclooxygenase enzymes. Proc Natl Acad Sci USA. 1993;90(15):7240—4.

40. Amin AR, Vyas P, Attur M, Leszczynska-Piziak J, Patel IR, Weissmann G, Abramson SB. The mode of action of aspirin-like drugs: effect on inducible nitric oxide synthase. Proc Natl Acad Sci USA. 1995;92(17):7926-30.