

Anti-inflammatory effects of three kinds of traditional Mongolian medicine monomer and its combination on LPS-stimulated RAW264.7 macrophages

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Abstract. – OBJECTIVE: Traditional Mongolian Medicine (TMM) exhibits useful biological activities including antifungal, antibacterial, and anti-inflammatory actions. The mechanisms of TMM in anti-inflammation were still unclear. The aim of this study was to investigate the effects of the three main monomers (geniposide, galate, berberine hydrochloride and a mixture of them) of a traditional Mongolian medicine on cell survival and the proinflammatory cytokines signaling pathways which are activated by bacterial lipopolysaccharides (LPS).

MATERIALS AND METHODS: Mouse macrophage-like cell line RAW264.7 was used as a model of inflammation to investigate the anti-inflammatory effects of three TMM monomers and their combination. RT-PCR and Western blot was used to quantify the change of mRNA and protein levels of cytokines, Toll-like receptor-4 (TLR4) and Nuclear Factor- κ B (NF- κ B) and its inhibitor I κ B. The non-radioactive electrophoresis mobility shift assay (EMSA) was used to evaluate the binding activity of NF- κ B.

RESULTS: The monomers and their combination exhibited a potent anti-inflammatory effect for suppressing the LPS-evoked secretion of proinflammatory cytokines IL-1 β , IL-6 and TNF α . Furthermore, the monomers and their combination attenuated activation of NF- κ B and expression of TLR4 at both mRNA and protein levels, the upstream player of the LPS-TLR4-cytokines/NF- κ B signaling pathway.

CONCLUSIONS: The Mongolia herbal compound exerts a potent anti-inflammatory effect and could potentially be developed as a useful agent for the chemo-prevention of inflammatory diseases.

Key Words:

Anti-inflammation, Traditional Mongolian medicine, Cytokines, NF- κ B, TLR 4, LPS.

Introduction

Inflammation is a protective physiological response of the body to activate the immune system in response to a variety of stimuli, including infections and tissue injury^{1,2}. Inflammatory diseases are related to the over-production of pro-inflammatory cytokines such as interleukin (IL)-6 and IL-1 β . Nitric oxide (NO) is also generated by inducible nitric oxide synthase (iNOS)³⁻⁵. Therefore, it is believed that the suppression of these inflammatory mediators is an important target for treating chronic inflammatory diseases. Inflammation is also a complex process mediated by the action of various immune cells, including natural-killer cells, neutrophils and macrophages. Macrophages play a central role in the inflammation process by mediating many different immunopathological phenomena, such as the over-expression of pro-inflammatory cytokines and inflammatory mediators. Hence, the macrophage cell line provides an excellent model for drug screening and evaluation of potential inhibitors of the inflammatory response.

Nonsteroidal anti-inflammatory monomers (NSAIDs) are the most broadly used medicines for treating inflammation-related diseases. However, the long-term administration of potent NSAIDs can result in various and severe adverse effects. During the past few years, there has been a resurgence of interest in isolating and developing naturally occurring monomers from medicinal plants⁶. Although traditional Chinese herbs have been used for centuries as remedies in China, their clinical uses are limited because we have not isolated and clinically tested all of the complex substances that make-up

these medicines. It is well known that many traditional Chinese herbs possess anti-sepsis function^{7,8} and traditional Chinese herbs have been often used for the treatment of various inflammatory diseases due to high effectiveness and minimal side effects. There is a Mongolia herb medicinal formula (MHMF) named “Te-Run-Su-Du-Le” which is used as an anti-inflammatory in Inner Mongolia. This Mongolian herb formula, with a history of over 2000 years, is similar to traditional Chinese herbs and can be used as a natural anti-inflammatory. There are 7 kinds of Mongolia herbs, including *Gardenia jasminoides*, *Rheum officinale* and *Coptis chinensis*, in this formula. It has been reported that Geniposide (isolated from *Gardenia jasminoides*), Epigallocatechin gallate (isolated from *Rheum officinale*) and *Berberine* (isolated from *Coptis chinensis*) play roles in inflammatory reactions⁹⁻¹¹. We propose that these three Mongolia medicine monomers or their combination may play major roles in anti-inflammatory effects in this MHMF.

In this study, we investigated the anti-inflammatory effects of these monomers (geniposide, gallate, berberine hydrochloride and their combination) on pro-inflammatory responses and the cytokine signaling pathways which are activated by bacterial lipopolysaccharide (LPS) challenge in the mouse macrophage-like cell line RAW264.7. It is well studied cell line for investigation of the inflammatory model. The levels of TNF α , IL-6, 1L- β and TLR4, and the NF- κ B dependent transcriptional activation induced by LPS were determined. TMM monomers and their combination repressed LPS-induced expression of these genes compared with pyrrolidine dithiocarbamate (PDTTC) and dexamethasone (DEX), which are well-known as a NF- κ B p65 inhibitor and an anti-inflammatory reagent, respectively. These monomers and their combination showed potent inhibitory activities on the production of these inflammatory factors.

Materials and Methods

Cell Culture and Reagents

RAW 264.7 cell line was obtained from China Center for Type Culture Collection. The cells were cultured in Dulbecco Modified Eagle's Medium (DMEM) (Gibco BRL Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum

(Gibco BRL Life Technologies Inc.) and maintained at 37°C in a humidified incubator containing 5% CO₂.

The effects of the principal monomers on the viability of RAW264.7 cells were examined. Cells were incubated in the compound Mongolian medicine and in each of these monomers separately, or in combination, at different concentrations for varying periods of time. Three different concentrations (designated low, medium, and high) of these monomers were determined. The concentrations of geniposide were 5, 10 and 15 mM, gallate 8, 16, and 32 μ g/L and berberine hydrochloride 10, 20, and 40 μ M. Drug incubation time ranged from 0-48 h.

Cell Viability Assay

Cell Counting Kit-8 (CCK-8; Shanghai Beyotime Inc, Shanghai, China) was used to quantify viable cells. RAW264.7 cells (5×10^3 cells/well) were seeded in 96-well plates and were incubated in a humidified incubator at 37°C with 5% CO₂ for 24 hours before experimental interventions. Upon completion of the 24-hour incubation the CCK-8 assay was performed according to the manufacturer's instructions. 10 μ l of CCK-8 solution was added to each well and plate was incubated at 37°C for 1 h. After shaking for 1 min, the absorbance was measured at 450 nm using a microplate reader. Each test condition was performed and measured in triplicate including control wells which contained cells in the absence of monomers. Additionally, a cell-free, drug-free well was measured for adjusting background noise.

Real Time PCR

Total cellular RNA was prepared from 10^6 cells using the RNeasy mini kit (Qiagen Valencia, CA, USA). The cDNA was synthesized using Invitrogen Superscript III (Invitrogen, Carlsbad, CA, USA). PCR reactions were carried out on the ABI-7900 RT-PCR with initial denaturation at 95°C for 10 min, 40 cycles of 95°C for 30 s, 60°C for 60 s and a final extension for 2 min at 60°C in a 50 μ l reaction mixture containing 2 μ l each cDNA, 0.2 μ M each primer, and 25 μ l 2X real-time master mix.

The threshold cycle number (CT) value for target genes were normalized against β -actin reference genes and calculated as Δ CT = CT_{target} - CT _{β -actin}. Relative target gene mRNA concentrations were expressed as multiples of it versus reference: F = 2 ^{Δ CT}.

Western Blot Analysis

Total protein samples from cultured RAW264.7 cells were extracted using the Total Protein Extraction Kit (ProMab, Richmond, CA, USA). Protein extracts were prepared in lysis buffer (50 mM Tris, pH 7.4, 1% NP-40, 150 mM NaCl, 1mM EDTA, 1 mM PMSF, 1 μ g/mL each of aprotinin, leupeptin, and pepstatin). Membrane protein samples from cell preparations were isolated using the ProteoExtract[®] Native Membrane Protein Extraction Kit (M-PEK; MERCK KGaA, Darmstadt, Germany). Nuclear and cytosolic protein samples were extracted from RAW264.7 cells using NucBuster TM Protein Exaction Kit (MERCK KGaA).

The intensity of bands in the Western blot and EMSA were obtained by the Gel-Doc system and analyzed with SigmaGel software (Jandel Scientific, San Rafael, CA, USA). The data of the Western blot were normalized to β -actin and presented as relative abundance.

Non-Radioactive Electrophoresis Mobility Shift Assay (Non-Rad EMSA)

Nuclear protein was extracted from the cell and analyzed for the presence of NF- κ B using a non-radioactive NF- κ B electrophoresis mobility shift assay (EMSA) kit (Viagene Biotech Inc, Tampa, FL, USA). Equal amounts of nuclear protein (5 μ g) from each sample were analyzed by EMSA following the manufacturer's instructions, as previously described¹².

The intensity of bands in EMSA was obtained by the Gel-Doc system and analyzed with SigmaGel software (Jandel Scientific, San Rafael, CA, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

Levels of IL-1 β , IL-6, and TNF- α in cell lysates were measured according to the manufacturer's instructions using the Mouse IL-1 β ELISA Kit, Mouse IL-6 ELISA Kit, and Mouse TNF- α ELISA Kit (Beyotime Inc, Shanghai, China), respectively.

Statistical Analysis

All of the data was expressed as mean \pm SD (standard deviation). Statistical analysis was performed with an analysis of variance (ANOVA) model for among group factor comparisons, and the Tukey HSD test was used for comparisons between the groups. All analysis was undertaken

using SPSS Version 10.0[®] software (SPSS Inc., Chicago, IL, USA) and $p < 0.05$ was considered to be statistically significant.

Results

Effects of the Monomers and the Monomers Combination on Viability of RAW264.7 Cells

We performed preliminary experiments to determine the optimal concentrations and length of time for each treatment. The concentrations of the monomers chosen were geniposide 15 mmol/L, gallate 32 μ g/L, berberine hydrochloride 40 μ mol/L and the monomer combination concentration grade consisted of a mixture of 1/3 of each monomer. The cells were incubated for 24 hours in our experiments. After the cells had been treated with monomers and their combination for 24 hours, they were then incubated with LPS (100 ng /ml)⁹ for 4 hours to induce pro-inflammatory responses.

The effects of the monomers and their combination on the cell viability are shown in Figure 1. Exposure of RAW264.7 cells to LPS leads to enormous cell death with the resulting cell viability of $63.6\% \pm 1.67\%$. The three kinds of monomers and their complex were all able to mitigate the decreases of cell survival induced by LPS challenge. The cell viability of the geniposide (GEN), gallate (GAL), berberine hydrochloride (BER), the complex of the monomers

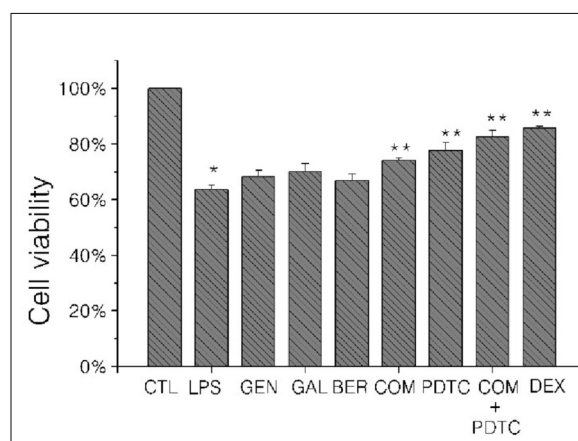


Figure 1. Effects of monomers and their combination on the cell viability in a cellular inflammation model induced by lipopolysaccharide (LPS) of the RAW264.7 cells. * $p < 0.05$ compared to CTL group, ** $p < 0.05$ compared to LPS group.

Table I. The following primer pairs use for real time PCR.

Gene	Forward primer	Reverse primer
P65	GCAGAAAGAAGACATTGAGG	TCATCTGTGTCTGGCAAGTA
TLR4	ACCTCTGCCTTCACTACAGA	AGGGACTTCTCAACCTTCTC
β -actin	GGACCTGACAGACTACCTCA	GTTGCCAATAGTGATGACCT

(COMP), pyrrolidine dithiocarbamate (PDTC) and dexamethasone (DEX) increased to $68.2\% \pm 2.33\%$, $70.00\% \pm 3.03\%$, $66.90\% \pm 2.32\%$, $74.10\% \pm 0.90\%$, $77.70\% \pm 2.81\%$, $82.60\% \pm 2.31\%$, $85.7\% \pm 0.57\%$ respectively. Compared to the three individual monomers the monomer complex has higher a cell survival rate. As expected, both the potent NF- κ B inhibitor PDTC (20 μ mol/L) and the anti-inflammatory and immunosuppressant dexamethasone (Dex, 10 μ g/ml) exhibited cytoprotection against LPS induced cell death similar to the test monomers and the strength of their action was in the same range as that of the monomers. Moreover, the cell viability of the co-application of PDTC and the monomer complex group is higher than that of the single PDTC group or the monomer complex group.

Effects of Mongolian Medicine Monomers on TLR4-NF- κ B Inflammatory Responses Signaling Pathway

The mRNA expressions of TLR4 and NF- κ B p65 were monitored to detect the involvement of transcriptional events during their biosynthesis. Changes in mRNA levels for TLR4 and NF- κ B

p65 were assessed by RT-PCR using β -actin as the control gene. As show in Figure 2, LPS promoted the transcription of both TLR4 and NF- κ B p65 sub-unit, as indicated by the elevation of the mRNA levels of TLR4 and NF- κ B p65. These up-regulations were moved towards the normal value under control conditions by all test monomers and positive control agents. The greatest magnitude of action was found to be elicited by the monomer complex, relative to the monomers. Again the complex and PDTC in combination demonstrated synergistic effects, particularly in the case of TLR4. Transcript levels of NF- κ B p65 and TLR4 were determined by real-time RT-PCR. Note that LPS induced substantial up-regulation of NF- κ B p65 and TLR4, and test monomers and positive control agents alleviated the up-regulation.

We performed Western blot analysis utilizing specific primary antibodies to detect the effects of the individual three monomers on protein levels of phosphorylated nuclear factor of light polypeptide gene enhancer in B-cells inhibitor (p-I κ B), NF- κ B p65, phospho-p65 (p-p65) and TLR4 in LPS-stimulated RAW 264.7. β -actin was used as loading control (Figure 3). p-I κ B-p-

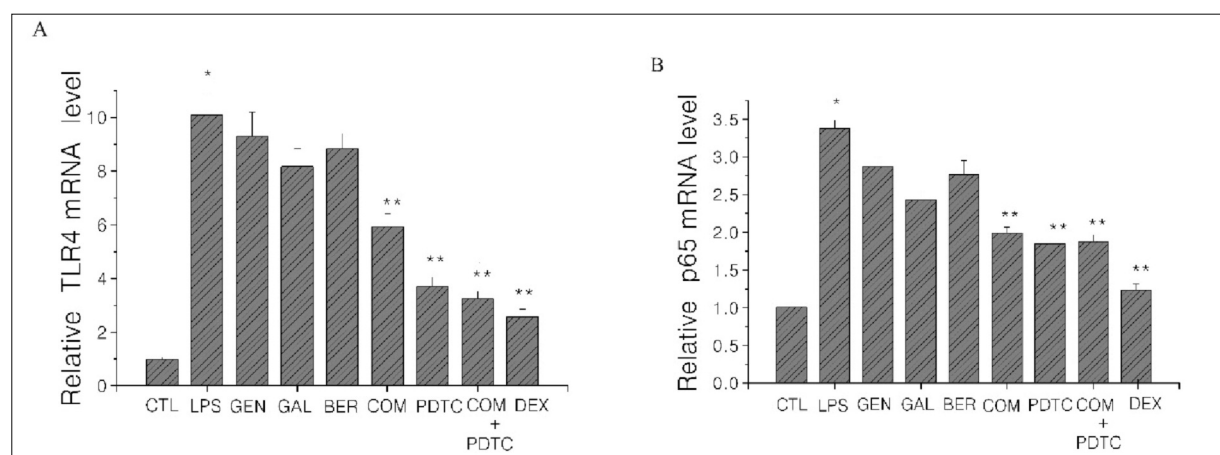


Figure 2. Repressive effects of Mongolian Medicine monomers on expression of NF- κ B p65 subunit (p65) and Toll-like receptor 4 (TLR4) in LPS-stimulated RAW 264.7. P65 **(A)**, TLR4 **(B)**. * $p < 0.05$ compared to CTL group, ** $p < 0.05$ compared to LPS group (n=3.)

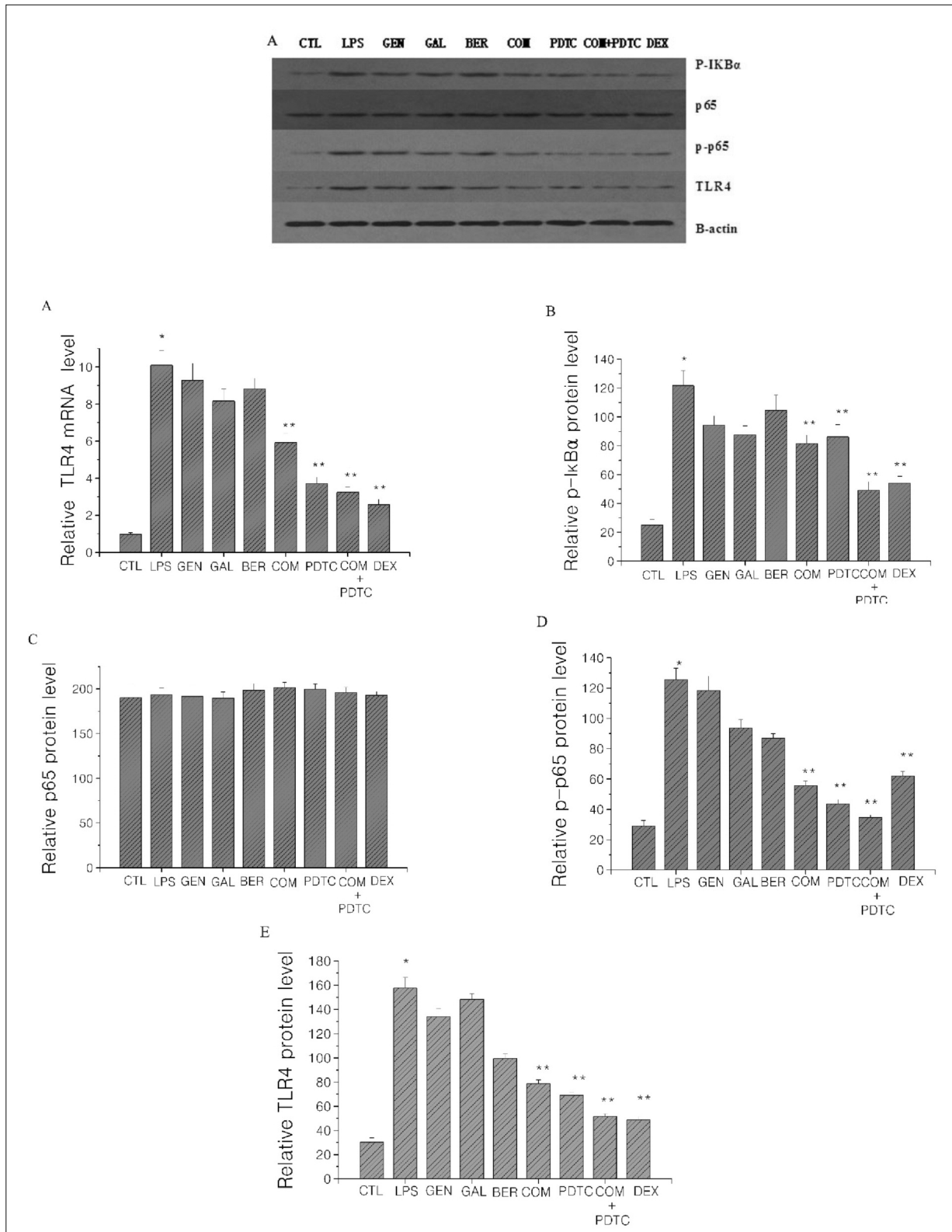


Figure 3. **A**, Effect of three kinds of Mongolian Medicine monomers on expression of p-IkB, p65, p-p65 and TLR4 proteins in LPS-stimulated RAW 264.7. Typical result of Western blot showed p-IkB, p65, p-p65 and TLR4 in LPS-stimulated RAW 264.7 (**A**), semi-quantitative analysis indicated the changes in levels of p-IkB (B), p65 (C), p-p65 (D) and TLR4 (E) (β -actin was used as control) respectively ($p < 0.05$, compared to CTL group, ** $p < 0.05$, compared to LPS group).

p65 and TLR4 were increased by LPS and were slightly attenuated by the individual three monomers and were significantly attenuated by the MMM mixture and positive control agents. Interestingly, the p65 protein level was not affected by LPS or the other treatments.

Non-radioactive electrophoresis mobility shift assay (EMSA) was conducted using the fragment encompassing the putative p65 *cis*-acting element to bind NF- κ B p65 protein in the nuclear extract from RAW264.7 cells (Figure 4). EMSA analysis demonstrated that three kinds of MMM slightly diminished and their mixture markedly diminished the enhancement of the binding between NF- κ B p65 and the putative p65 *cis*-acting element which was induced by LPS. The NF- κ B p65 DNA binding activity was parallel to NF- κ B p65 protein level.

Inhibitory Effects of Monomers and the Monomers Complex on LPS-Induced Secretion of Proinflammatory Cytokines

LPS significantly ($p < 0.001$) increase the amounts of IL-1 β , IL-6 and TNF- α in RAW264.7 cells (Figure 5). The monomers as well as the positive control agents (PDTC and Dex) all significantly mitigated the LPS-induced secretion of the pro-inflammatory cytokines. Notably, the monomer complex elicited the greatest effect among the test monomers and co-application of the monomer complex and PDTC elicited synergistic effects: the magnitude of reduction with the combination of 3 monomers was greater than that with each of them alone. For example,

the TNF- α content of the single monomer complex and PDTC was 405.33 and 390.12 pg/mL respectively. While the TNF- α content of combined application of monomer complex and PDTC decreased to 253.68 pg/mL. The observed anti-inflammatory efficacy was in good agreement with the cytoprotective actions of the test monomers.

Discussion

Inflammation is a complex process regulated by a variety of immune cells and effector molecules. IL-6, IL-1 β and other proinflammatory cytokines are important mediators of macrophage-mediated inflammation. The expressions of IL-6, IL-1 β and other proinflammatory cytokines are regulated by NF- κ B. It has been reported that TLR4 and mitogen-activated protein kinases (MAPKs) can affect the activity of NF- κ B¹³. Therefore, the inhibition of these mediators with pharmacological modulators may be an effective therapeutic strategy for preventing inflammatory reactions and diseases.

Macrophage activation is important in the progression of multiple diseases through the release of inflammatory mediators. LPS is known to act as the prototypical endotoxin because it binds the CD14/TLR4/MD2 receptor complex, which promotes the secretion of proinflammatory cytokines in macrophages and B cells through transcription factor NF- κ B. NF- κ B is located in the cytoplasm and binds with its inhibitory protein I κ B in un-

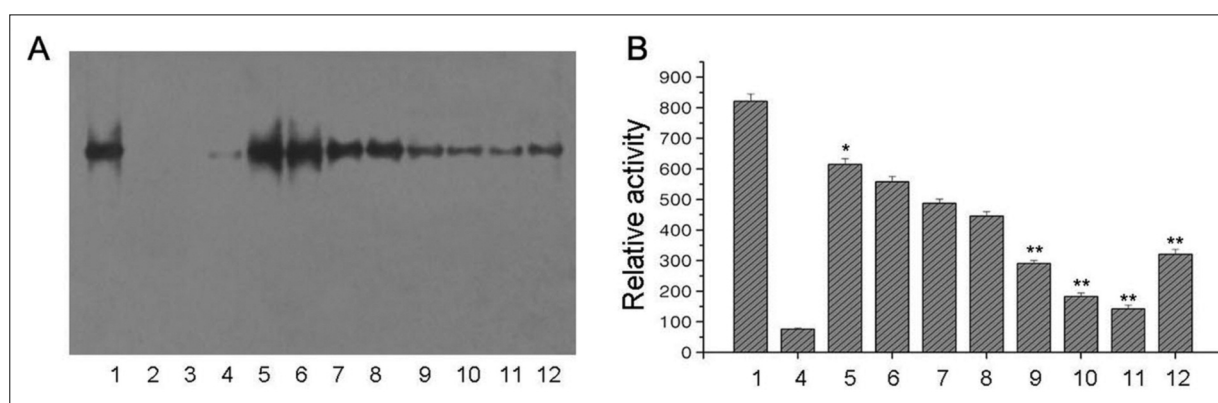


Figure 4. Suppressive effects of Mongolian Medicine monomers on the transcriptional activities of NF- κ B p65 subunit in the nuclei of RAW264.7 cells. **(A)** A typical example of image showing the shifted band representing the DNA-p65 complex using non-radioactive electrophoresis mobility shift assay (EMSA). **(B)** Digital data of the bands shown in the (A) panel. (Lane 1: positive control; Lane 2, 3: 100-folds unlabeled probe; Lane 5: LPS; Lane 6: Gal; Lane 7: Gal; Lane 8: Ber; Lane 9: Com; Lane 10: PDTC; Lane 11: PDTC+Comp; Lane 12: Dex.). * $p < 0.05$, compared to CTL group, ** $p < 0.05$, compared to LPS group.

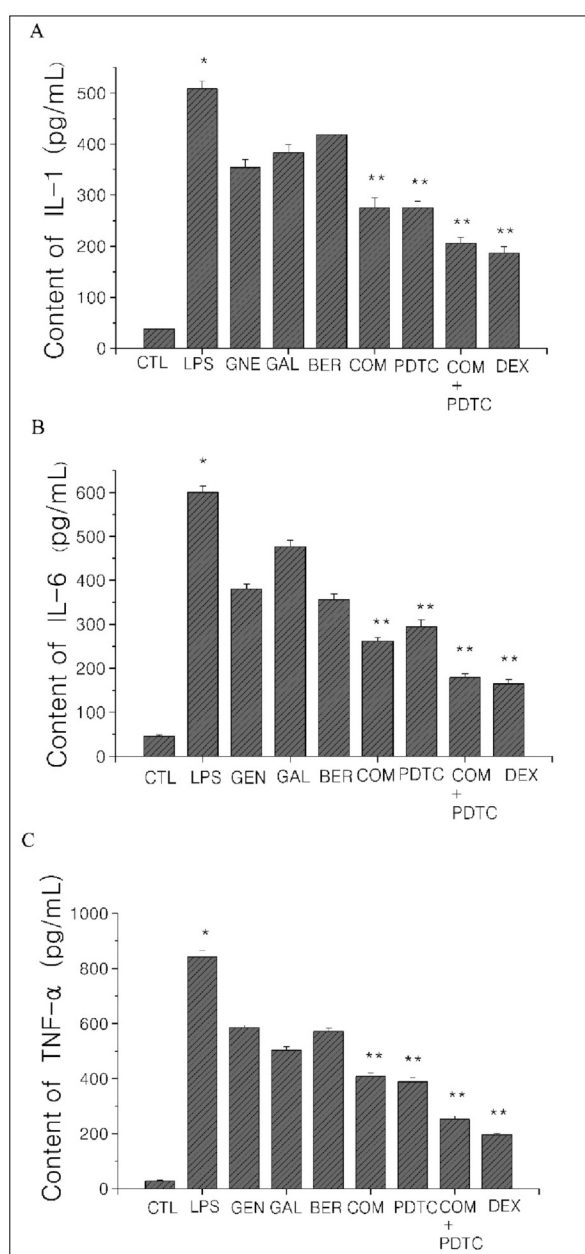


Figure 5 LPS-induced elevation of intracellular levels of potent proinflammatory cytokines interleukin IL-1 β , iIL-6 and TNF- α in RAW264.7 cells. **(A)** IL-1 β **(B)** IL-6 **(C)** TNF- α . (* p < 0.05 compared to CTL group, ** p < 0.05 compared to LPS group).

stimulated cells. When the cells are exposed to the stimulants such as LPS, I κ B is phosphorylated and liberates NF- κ B, resulting in NF- κ B translocation into the nucleus. Nuclear NF- κ B, then, binds to the promoters of pro-inflammatory mediators, resulting in the induction of their gene expression¹⁴⁻¹⁷. LPS is widely used to elucidate the

anti-inflammatory effects of some compounds in RAW264.7 macrophages^{12,18-28}.

In order to understand the anti-inflammatory compounds from plants in Mongolia herb medicinal formula "Te-Run-Su-Du-Le", the effects of three kinds of MMM on TLR4/NF- κ B inflammatory responses signaling pathway were explored using RAW264.7 macrophages treated with LPS. Zheng et al⁹ showed that geniposide can directly bind LPS and neutralize it *in vitro*. Geniposide exerted an anti-inflammatory effect by regulating TLR4 expression, which affected the downstream NF- κ B and mitogen-activated protein kinase (MAPK) signaling pathways. The inhibitory effect of epigallocatechin-3-gallate is largely related to the ability of the molecule to down-regulate the inflammatory mediators (iNOS and COX-2) biosynthesis at a transcriptional level²⁹. Jeong et al³⁰ showed that BER would down-regulate pro-inflammatory responses in macrophages via AMPK stimulation. The inhibitory effects of BER on proinflammatory gene expression and signaling cascades were abrogated by inhibition of MAPK activity. Berberine can act as a LPS antagonist and block the LPS/TLR4 signaling from the source, resulting in the anti-bacterial action¹¹. In this study, the mixtures of three kinds of MMM down-regulated the expression of TLR4 at both transcriptional and post-transcriptional levels and reduced the NF- κ B binding activity that was induced by LPS. At the same time, the combination of 3 monomers may be a more efficient anti-inflammatory due to their different inhibition site in the TLR4/NF- κ B signaling pathway.

It is clear that both TLR4 and MAPK are involved in the expression of IL-1 β , IL-6 and TNF- α which are regulated by NF- κ B¹³. TLR4 is one of the well-characterized pathogen recognition receptors (PRRs) that recognize LPS of Gram-negative bacteria³¹⁻³³. TLR4 which plays a central role in the regulation of the host immune system, was found to be down-regulated by the three monomers and their combination in this study. We did not detect any change in MAPK activity. Our experiments provided several pieces of evidence in support of the notion that the MMM components are able to prevent activation, but not expression, of NF- κ B p65, as indicated by the reduced phosphorylated form of NF- κ B-p65 and weakened NF- κ B p65 DNA binding capacity. Due to the change of NF- κ B p65 DNA binding capacity, NF- κ B target genes such as IL-1 β , IL-6 and TNF- α , were reduced by MMM when induced by LPS.

Conclusions

The present study provides preliminary evidence that traditional Mongolian medicine monomers and their compounds have anti-inflammatory effects on LPS-stimulated RAW264.7 macrophages. The Mongolia herbal compound exerts a potent anti-inflammatory effect and could potentially be developed as a useful agent for the chemoprevention of cancer or inflammatory diseases.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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