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Suppression of Inflammatory Macrophage Responese by *Glycyrrhiza Uralensis* Herbal Acupuncture Extract

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감초 약침액이 대식세포주에서 항염증효과에 미치는 영향

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Abstract

목적 : 본 연구는 감초의 염증에 대한 효과를 연구하였다. 감초의 에탄올 그리고 물로 추출한 두 가지의 약침액을 이용하여 쥐의 대식세포에서 유래한 RAW264.7 세포에 대한 염증 억제효과를 확인하였다.

방법 : Inducible nitric oxide synthase(iNOS), cyclooxygenase-2(COX-2)를 포함한 염증성 단백질의 발현과 extracellular signal-regulated kinase 1/2(ERK1/2) 그리고 phosphorylated ERK1/2 의 발현을 Western blot 으로 확인하였고, PGE2의 발현은 ELISA 로 확인하였다.

결과 : RAW264.7 세포에 감초의 물 혹은 에탄올 추출 약침액을 투여한 결과 투여된 농도에 따라 LPS로 유도된 NO의 생성이 억제되었으며 iNOS, COX-2, 그리고 인산화 ERK1/2 의 발현도 감소되었다.

결론 : 본 실험 결과, 적작약의 물 그리고 에탄올 추출 약침액에 대하여 항염증성 효과가 있음을 확인하였다.

중심단어 : *Glycyrrhiza uralensis*, anti-inflammation, ERK1/2, iNOS, Cox2

Ⅰ. Introduction

Pharmacupuncture is unique treatment combined pharmacotherapy with acupuncture in oriental medicine. recently study was reported that *G. uralensis* pharmacupuncture using water extract from *G. uralensis* showed inhibition of anti-allergic effect[1].

G. uralensis belongs to the Leguminosae family, and is used as a traditional medical herb in oriental medicine. Recently studies have demonstrated *G. uralensis* being used to treat liver, asthma, and other diseases[2,3]. Another study suggests that it has anti-inflammatory properties[4]. However, for

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the treatment of inflammation, further studies, including the elucidation of the molecular mechanism, are necessary. It was reported that MeOH extract from *G. uralensis* was has anti-inflammatory effect on mouse macrophages[4], whereas other extracts yet. We examined anti-inflammatory effects of EtOH extract form *G. uralensis* and compared with effects of water extract.

Inflammation is implicated in the pathogenesis of many diseases and is involved in secretion of inflammatory mediators and cytokines such as reactive oxygen species (ROS), reactive nitrogen species, nitric oxide and prostaglandin E2 (PGE2)[5-9]. Macrophages are proposed to play a central role in the inflammatory response, and serve as an essential interface between innate and adaptive immunity[10]. Macrophages exert their anti-microbial effects directly through phagocytosis[11]. In the inflammatory process, during exposure to lipopolysaccharide (LPS) or interferon-γ (IFN-γ), macrophages are activated and release pro-inflammatory mediators and cytokines including NO, ROS, PGE_2, and others[12,13]. Overproduction of inflammation mediators causes many diseases such as asthma, Alzheimer's disease and atopic dermatitis[14-17]. Previous studies have demonstrated that inhibition of pro-inflammatory mediators secreted by macrophages could attenuate the severity of these disorders.

Nitric oxide (NO) production is a diffusible intercellular molecule and also an intracellular signaling molecule[18]. NO has diverse effect on the physiological function of smooth muscle cells, neurons, platelets and immune cells. Nitric oxide synthases (NOS) generate NO by catalyzing the oxidation of guanidine-nitrogen of L-arginine[19,20]. NOS has three different isoforms that are expressed in a tissue-specific manner: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS)[21]. nNOS and eNOS are stimulated to synthesize NO by the calmodulin signaling pathway[22]. iNOS is normally not detected but can be up-regulated in a variety of tissues and cells after stimulation with lipopolysaccharide (LPS) or cytokines[23,24]. High levels of NO production from iNOS during the inflammatory response is detrimental to cell viability and function[25]. NO is a free radical and its cytotoxic effects are due to peroxynitrite (OONO-) formation and nitration of tyrosine residues in proteins, or generation of superoxides[26].

Cyclooxygenase (Cox), a prostaglandin synthase, is the rate-limiting step in the synthesis of biologically active and physiologically important prostaglandins[27]. There are two isoforms of Cox; Cox1 and Cox2. Cox1 is constitutively expressed in most tissues and, in contrast, Cox2 is expressed in low or undetectable levels in the resting-state of cells[28,29]. Cox2 expression is induced by inflammatory cytokines and tumor promoters. Cox2 expression is regulated by several pathways[30] and both the Akt and MAPK pathways seem to play an important role[31].

In this study, we prepared two types of extracts from *G. uralensis*, using a water-based or ethanol-based extraction method. We evaluated the effect of each extract for anti-inflammatory activity and cytotoxicity. Our results suggest that *G. uralensis* has anti-inflammatory properties that are mediated by the inhibition of iNOS and Cox2 expression and extracellular signal-regulated kinase (ERK1/2) phosphorylation.

II. Materials and Methods

1. Preparation of Plant Extracts

G. uralensis was extracted exhaustively with 70% ethanol at 70 ℃ and distilled water (DW) at 100 ℃ for 4 h. The extracted sample was filtered and evaporated under reduced pressure at 40 ℃ in a vacuum rotatory evaporator. Afterwards, the concentrated mass was dried and weighed to determine the total extractable compounds. Finally, prepared extract was stored at 4 ℃ until use. The percentage of yield (w/w) was calculated as [Yield (%) = (total extracted sample mass / total dry sample mass) × 100]. Stock solutions were prepared by dissolving the extracted mass in DW.

2. Cell Culture

RAW264.7 (mouse macrophage) cell line was obtained from American Type Culture Collection (ATCC, USA). RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO) and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin; GIBCO) at 37 ℃ in 5% CO_2.

3. MTT Assay

1 x 10^5 cells were seeded in each well of a 96-well plate and incubated for 16 h at 37 ℃ in 5% CO_2. The cells were then exposed to *G. uralensis* at various concentrations for 16 h and followed by addition of MTT formazan substrate. After 2 h of incubation at 37 ℃, the supernatant was discarded and 100 ㎕ of DMSO was added. The optical density was measured at 570 nm lengths using a spectrophotometer (SPECTRA190, Molecular Devices). Cell survival rates were expressed as a percentage of the value of the medium (control) group.

4. NO Release Assay

The cells were cultured at a density of 1 x 10^5 cells per well in a 96-well plate format and incubated with LPS at a concentration of 1㎍/㎖. After 16 h, the medium was harvested and NO production was measured using the Griess reagent. One hundred percent activity was defined as the difference between NO formation in the absence (blank) and in the presence (control) of LPS for 20 h, performed in triplicate. The percent inhibition was calculated as [1 - (NO level of sample - NO level of blank)/(NO level of control - NO level of blank)] x 100.

5. Measurement of PGE_2

Quantitation of protein secretion was performed by ELISAs according to the manufacturer's instruction. RAW264.7 cells were cultured in 6-well plates and incubated in the presence or absence of LPS (1 ㎍/㎖) for 16h. Next, the culture medium supernatant was collected to determine PGE_2 concentration and quantitation was performed with a spectrophotometer (SPECTRAMAX190, Molecular Devices). Values from the proliferation assays were used as the standard.

6. Immunoblotting Assay

RAW264.7 cells were incubated overnight in 6-well tissue culture plates and incubated further in DMEM without L-glutamate for 24 h. The cells were washed and lysed in a homogenizing buffer containing protease inhibitors. 25 ㎍ of total cell lysates were resolved by SDS-PAGE and transferred to a PVDF membrane. After blocking for non-specific antibody binding (5% BSA,

TBS-T), the blots were incubated overnight with diluted (1%BSA TBS-T) antibodies specific for Cox2 (1:1000 dilution; Cell Signaling Technology), iNOS (1:2000; Becton-Dickinson), Erk1/2 (1:1000; Cell Signaling Technology), or phosphorylated Erk1/2 (1:1000; Cell Signaling Technology). After several washes in TBS-T, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase specific for mouse or rabbit IgG (1:20000 dilution) for 1 h at room temperature. Following three washes in TBS-T, immunoreactive bands were visualized by enhanced chemiluminescence (LI-COR, Odyssey).

III. Results

1. Effect of water extract of *G. uralensis* on cytotoxicity and NO production

We examined whether the water-based extract of *G. uralensis* is cytotoxic. RAW264.7 cells were exposed to different concentration of the extract at 1, 5, 25, 125, 625, and 3125 ㎍/㎖ for 16 h. The MTT assay revealed cytotoxicity at concentrations of 625 ㎍/㎖ and 3125 ㎍/㎖. To verify the anti-inflammatory activity we measured NO production and found decreased expression at 5 to 3125 ㎍/㎖ of *G. uralensis* extract (Fig. 1). Interestinlgy, the increased cytotoxicity of the *G. uralensis* extract correlated with a decrease in NO release. These results suggest that the water-based extract prepared from *G. uralensis* has anti-inflammatory effects because of the decrease in NO production even at concentrations as low as 125 ㎍/㎖.

2. Effect of ethanol extract of *G. uralensis* on cytotoxicity and NO production

We also determined whether the ethanol-based extract of *G. uralensis* is cytotoxic. RAW264.7 cells were treated with 1, 5, 25, 125, 625, 3125 ㎍/㎖ of ethanol extract for 16 h and cell toxicity was observed at a concentration of 3125 ㎍/㎖, as determined by MTT assay. Next, we asked whether the ethanol extract of *G. uralensis* has anti-inflammatory properties as seen with the water extract. We observed a decrease in NO production starting at 5 ㎍/㎖ of extract (Fig. 1) without any cytotoxicity. These results suggest that the ethanol-based extract of *G. uralensis* has a more potent anti-inflammatory activity than the water-based extract.

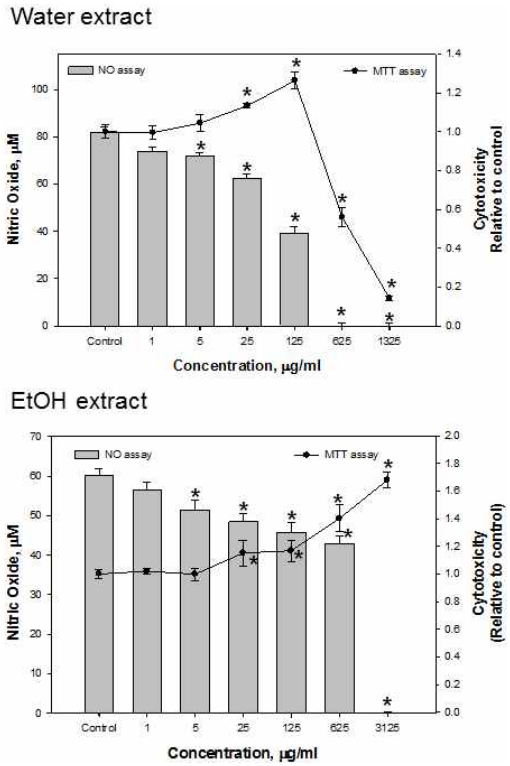

Fig. 1. Inhibitory effect of EtOH and water extracts from *Glycyrrhiza uralensis* on nitric oxide production in LPS-stimulated RAW264.7 cells.
The cells (1.0×10^5 cells/well) were stimulated with 1

㎍/㎖ of LPS or with LPS plus various concentrations (1, 5, 25, 125, 625, 3125 ㎍/㎖) of ethanol-based or water-based *Glycyrrhiza uralensis* extract for 24 h. Nitric oxide production was determined using the Griess reagent method. Cell viability was determined 24 h after the cells (1.0×10^4 cells/well) were stimulated with LPS (1 ㎍/ml) and extract. Cell viability was determined using the MTT method. Values are presented as the mean ± S.D. of experiments performed in triplicate (*$p < 0.05$).

3. Effect of extracts of *G. uralensis* on expression of PGE_2

Upregulation of Prostagrandin E_2 (PGE_2) expression is known to be related to inflammatory activity. We investigated the effect of each extracts from *G. uralensis* (50, 100, 150, 200 ㎍/㎖) on the expression of PGE_2. When RAW264.7 cells were stimulated by LPS they released PGE_2; however, PGE_2 production was suppressed slightly by both the water and ethanol extracts of *G. uralensis* at a concentration of 150 ㎍/㎖.

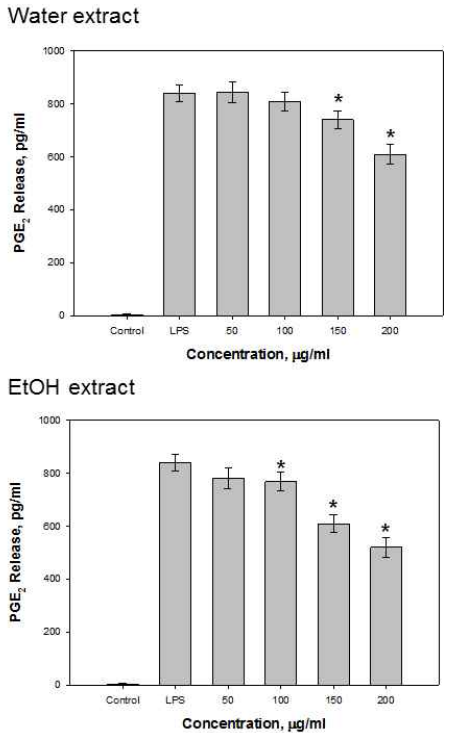

Fig. 2. Inhibitory effect of EtOH and water extracts from *Glycyrrhiza uralensis* on PGE_2 production in LPS-stimulated RAW264.7 cells.

The cells (1.0×10^4 cells/well) were stimulated with 1 ㎍/㎖ of LPS or with LPS plus various concentrations (50, 100, 150, 200 ㎍/㎖) of ethanol-based or water-based *Glycyrrhiza uralensis* extract for 16 h. PGE_2 production and release into the culture medium was assayed by ELISA. The data represent the mean ± S.D. of experiments performed in triplicate (*$p < 0.05$).

4. Inhibition of NO production and suppression of ERK1/2 phosphorylation by the water and ethanol extracts of *G. uralensis*

LPS-induced RAW264.7 cells release NO in a process mediated by the iNOS system. We determined the expression levels of pro-inflammatory signaling molecules after treatment with the *G. uralensis* extracts. Cox2 and iNOS expression were low or undetectable in the resting, non-induced state. However, when NO production was induced by LPS-stimulation, Cox2 and iNOS expression increased and became suppressed when the cells were treated with either extract at a concentration of 150 or 200 ㎍/㎖ (Fig. 3). Similarly, ERK1/2 was phosphorylated upon LPS stimulation and ERK1/2 phosphorylation was found to be inhibited after treatment with each of the extracts (Fig. 4). Together, these results suggest that extracts prepared from *G. uralensis* have anti-inflammatory effects by inhibiting certain pro-inflammatory molecules.

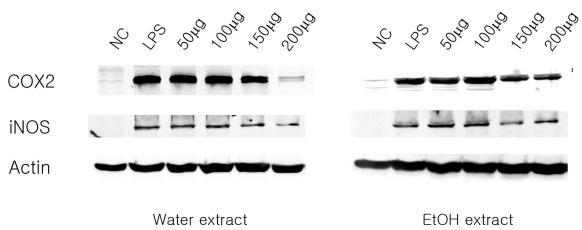

Fig. 3. Inhibitory effects of EtOH and water extracts from *Glycyrrhiza uralensis* on iNOS and Cox2 expression in LPS-stimulated RAW264.7 cells.

RAW264.7 cells (1.0×10^6 cells/ml) were stimulated with LPS (1 ㎍/㎖) in the presence of extract (50, 100, 150, 200 ㎍/㎖). Whole cell lysates (50 ㎍) were prepared and subjected to 10% SDS-PAGE. Expression of iNOS, COX-2 and actin were determined by Western blot analysis. Actin was used as a loading control.

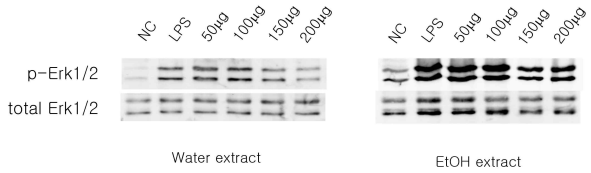

Fig. 4. Inhibitory effect of EtOH and water extracts from *Glycyrrhiza uralensis* on ERK1/2 Phosphorylation in LPS-stimulated RAW264.7 cells.
RAW264.7 cells (1.0×10^6 cells/ml) were stimulated with LPS (1 ㎍/㎖) in the presence of extract (50, 100, 150, 200 ㎍/㎖). Whole cell lysates (50 ㎍) were prepared and subjected to 10% SDS-PAGE. The expression of phosphorylated ERK1/2 was determined by Western blot analysis and the total cellular ERK1/2 was used as the loading control.

IV. Discussion

G. uralensis is an herbal plant that is used in oriental medicine. Recently study reported anti-inflammatory properties of glycyrol which is methanol extracts from *G. uralensis*[4]. Whereas, effect of other extract from *G. uralensis* is yet to be reported. Thus, we investigated anti-inflammatory effect of EtOH and water extract. Our data suggest that the pharmacological activity of *G. uralensis* is due to the inhibition of inflammation mediators and cytokines such as NO, iNOS and PGE_2 in mouse macrophages. Specifically, these inhibitory effects were mediated by inhibition of iNOS activation and the MAPK pathway.

Macrophages, found in most tissues and organs, have critical roles in immunity including phagocytosis and secretion of proinflammatory cytokines[8,10]. Macrophages are activated by bacteria and secrete inflammation mediators including vascular amines, arachidonic acid metabolites, proinflammatory cytokines, PGE_2 and reactive oxygen species (ROS). RAW264.7 cells, a mouse macrophage cell line, generate NO when stimulated by cytokines or LPS. Therefore, we examined the anti-inflammation and anti-oxidation potential of *G. uralensis* extracts using LPS-challenged RAW264.7 macrophages.

Some recent studies have shown that plant extracts can inhibit the generation of inflammation mediators (NO, PGE_2, iNOS and IL-6) in macrophages. These plant species include Astragalus membranaceus, Ostericum koreanum, and others[32,33].

Many studies have reported that LPS activates macrophages and induces Cox2 expression, an enzyme that of converts arachidonic acid into PGE_2. Recently, studies have shown that Cox2 induction is associated with cell toxicity because inhibition of Cox2 expression and/or activity reduces brain injury after ischemia and slows the progression of Alzheimer's disease and Parkinson's disease[34-36]. Nitric oxide has high reactivity as a free radical and has an important role physiologically for immune reaction in low concentrations (nanomolar range)[37], but high concentrations (micromolar range) of NO result in many pathological process including inflammation[38]. In mammalian cells, NO production is generated from three different isoforms of NOS; endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS)[21]. It has been reported that iNOS is not normally expressed, but LPS up-regulates iNOS expression in RAW264.7 cells. Exclusive release of NO production was caused by expression of iNOS in macrophages stimulated by LPS and other proinflammatory cytokines and was correlated

with several disorders[23,24,38]. Generally, expression of iNOS is induced by immune reaction and plays a role in exacerbating inflammation. In our study, *G. uralensis* significantly suppressed LPS-induced NO production in RAW264.7 cells in a concentration-dependent manner. Moreover, this suppression did not lead to cytotoxicity.

PGE_2, an important mediator of the inflammation, is synthesized by Cox2 and excess production causes inflammatory diseases such as Alzheimer's disease, Parkinson's disease and colon cancer. Suppression of PGE_2 production relieves inflammation and pain. Our results show that LPS-stimulated PGE_2 was suppressed by *G. uralensis* extracts in RAW264.7 cells; however, the decrease was slight. Importantly, this inhibition was concentration-dependent and did not exhibit any cell-toxicity. Other intracellular signaling pathways, including the MAPK pathway, are needed to induce and maintain the inflammatory process. The MAP kinases regulate Cox2, iNOS, and proinflammatory cytokines in LPS-stimulated macrophages[39]. Similar to PGE_2, *G. uralensis* extracts also inhibited the phosphorylation of ERK1/2 in a dose-dependent manner.

In conclusion, we present evidence that the water and ethanol extracts reduce inflammation by inhibiting NO production and PGE2 and ERK1/2 phosphorylation. In the case of the water extract, high concentrations (greater than 625 ㎍/㎖) are cytotoxic. Although the two extraction methods exhibit slightly different cytotoxicity, they have similar efficacy in reducing inflammation. Therefore, extracts prepared from *G. uralensis* may prove to be an effective therapy for other inflammatory diseases.

Ⅴ. Conclusions

Our data indicate that the extracts prepared from *G. uralensis* have anti-inflammation properties and exert their effects by inhibiting NO and PGE_2 production and ERK1/2 phosphorylation. In the case of the water-based extract, however, excessive concentration can be cytotoxic. Therefore, the ethanol-based extract prepared from *G. uralensis*, which has a slightly different toxicity but similar efficiency on inflammatory activity, might be the preferred method of preparing extracts of *G. uralensis*. Future studies might prove *G. uralensis* to be an effective therapy for many other inflammatory diseases.

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