The Effect of *Woogakseungmatang* Extract on NO Production in LPS- Stimulated RAW 264.7 Cells

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우각승마탕이 LPS로 유도된 RAW 264.7 세포에서 NO 생산에 미치는 영향

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Objectives : Woogakseungmatang is a prescription medication mainly used to treat facial paralysis in Korean medicine. The purpose of this study is to investigate the effects of Woogakseungmatang on anti-inflammation and anti-oxidation. **Methods :** Woogakseungmatang was extracted using hot water. Cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) method; nitric oxide(NO) production and Prostaglandin E₂(PGE₂) production in RAW cells treated with Woogakseungmatang were investigated; and the cytokine changes associated with inflammation were examined. The antioxidant capacity of Woogakseungmatang was measured using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. **Results :** RAW cells treated with Woogakseungmatang showed 90% cell viability at a 100- μ g/ml concentration. NO production was decreased by 15% at a 100- μ g/ml concentration. PGE₂ production was decreased by 18% at a 100- μ g/ml concentration. Interleukin 1 β (IL-1 β), interleukin 6(IL-6), and tumor necrosis factor- α (TNF- α) were significantly reduced at 100 μ g/ml compared with those in the control group. The DPPH free radical scavenging capability was more than 50% at 100 μ g/ml. **Conclusions :** Woogakseungmatang showed only a slight anti - inflammatory effect at 100 μ g/ml and it was difficult to confirm the concentration-dependent anti-inflammatory effect. Therefore, this study means to confirm the potential anti-inflammatory effects of Woogakseungmatang. Based on this research, more systematic and diverse studies should be conducted.

Key words : *Woogakseungmatang*, *Seogakseungmatang*, Anti-inflammation, Antioxidant, Macrophage, Korean Medicine

Introduction

An inflammatory reaction is a defensive reaction against a harmful stimulus to the living body. It is a defense mechanism of a living body that localizes the influence of an external

stimulus to the cells and tissues of the living body to restore and maintain a damaged region to normal¹⁾. However, if a defense mechanism is not performed smoothly in the human body and the inflammation reaction continues, it is a main factor in various inflammatory diseases, such as skin diseases,

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asthma, cancer, coronary artery disease, and rheumatoid arthritis²⁾. Therefore, the fast and effective defense against and elimination of inflammatory substances are essential for the prevention and treatment of various inflammatory diseases.

Woogakseungmatang consists of the substitution of Seogak (*Rhinocerotidae unicornis L.*) for Woogak (*Bubalus bubalis L.*) among the constituents of Seogakseungmatang. Seogak is the horn of a rhinoceros that has been cut and dried; the rhinoceros is an endangered species, and rhinos are protected by the Convention on International Trade in Wildlife Species, so these horns have been replaced with those of water buffalo.

Woogak is used to treat headaches, high fever, and skin diseases in Korean medicine; Woogak has similar efficacy to Seogak. In a recent clinical study, the use of Woogak rather than Seogak was effective for fever and pediatric fever; however, when Woogak is used instead of Seogak, its capacity must be increased. Generally, 30~120 g should be added to the daily intake capacity and boiled in water. In clinical practice, it is common to prescribe Woogak instead of Seogak³⁾.

In Donguibogam, Seogakseungmatang is used medicinally when the muscles are paralyzed and painful. It can be applied to the forehead, chin, and cheeks to treat facial pain and the paralysis of facial muscles. Therefore, Seogakseungmatang mainly uses facial pain for the same idiopathic paralysis⁴⁾.

Facial paralysis is the most common disease of the brain; it is caused by paralysis of the muscles of the eyes and mouth, and the symptoms can make a patient appear crooked on one side. The causes of this disease include thoughtfulness, overwork, and cold exposure. Symptoms can include senile neuropathy, sensory depression, abnormal sensation in the trigeminal nerve, facial numbness, pain, auditory irritability, larynx, and tinnitus⁵⁾. In the case of facial paralysis with severe pain, anti-inflammatory drugs are prescribed to reduce the inflammatory response⁶⁾. In Korean medicine, Woogakseungmatang is specifically prescribed for painful facial paralysis; therefore, it is necessary to study the anti-inflammatory effect of Woogakseungmatang.

Previous studies on Seogakseungmatang have been used in cases of trigeminal neuralgia⁶⁾ and antithrombotic studies⁷⁾. Studies on the anti-inflammatory effects of Woogakseungmatang are severely limited; we studied 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), nitric oxide (NO), Prostaglandin E₂ (PGE₂), and cytokine to determine the anti-inflammatory and 1,1-diphenyl-2-picrylhydrazyl (DPPH) effects of Woogakseungmatang.

Materials and Methods

1. Materials

The medicinal materials used in the experiments were purchased from OmniHub (Kyeongbuk, Korea). The prescription's composition was in accordance with Donguibogam (Table 1). Five batches of the prescription were placed in 3 L of water and subjected to hot water extraction for four hours. The filtrate was first filtered and then poured through filter paper. The filtrate was concentrated under reduced pressure in a rotary vacuum evaporator, and the concentrated solution was lyophilized in a free dryer for seven days. After drying, 26.53 g of concentrated product was obtained (yield: 7.07%). The obtained powder was stored at -80°C in a cryogenic freezer and diluted to the required concentration according to the experiment.

Table 1. Compositions of Woogakseungmatang. Prescription was based on Donguibogam. 6 g of *Rhinocerotis Cornu* was replaced with 48 g of *Buffle Cornu*

Components	herbs of Woogakseungmatang	Content
	Buffle Cornu	48 g
	Cimicifugae Rhizoma	5 g
	Sapshnikoviae Radix	4 g
	Osterici Radix	4 g
	Cnidii Rhizoma	3 g
	Aconiti Koreani Tuber	3 g
	Angelicae Dahuricae Radix	3 g
	Scutellariae Radix	3 g
	Glycyrrhizae Radix	2 g
	Total	75 g

2. Cell culture

The RAW 264.7 cells used in the experiments were purchased from Korean Cell Line Bank. Separated cells were suspended in 20 ml of Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin and cultured in a cell incubator (37°C, 5% CO₂). There were 10 or fewer subcultures. Cells were subjected to adaptation times for 24 hours before the samples were treated.

3. Cytotoxicity

Cells were plated at 1×10^4 cells/well in a 96-well plate and cultured for 24 hours. The extracts were treated at 10, 25, 50, 100, and 200 $\mu\text{g/ml}$ for 24 h. After incubation, 20 μl of MTT (5 mg/ml) solution was added and incubated in a cell incubator (37°C, 5% CO₂) for 4 hours. The supernatant of the sample was removed, 100 μl of dimethyl sulfoxide (DMSO) was added, and it was shaken for 30 minutes. After the reaction, the absorbance at 570 nm was measured using an ELISA reader and the cell survival rate of the control group was expressed as a percentage.

4. NO production

Ninety six well plates at 1×10^4 cells/well were cultured for 24 hours. After cultivation, the cells were replaced with fresh culture medium. The cells were treated with 1 $\mu\text{g/ml}$ of LPS. The extracts were treated at 10, 25, 50 and 100 $\mu\text{g/ml}$. And cultured for 24 hours in a cell culture incubator. The supernatant of the sample and the Griess reagent were mixed in the same ratio. The mixed sample was shaken for 5 minutes. The absorbance was measured at 540 nm. The NO concentration was determined using the standard curve of the NaNO₂ standard, and the NO value for the control was expressed as a percentage.

5. PGE2 production

Cells were seeded at 1×10^5 cells/well in a 12 well plate and cultured for 24 hours. After cultivation, the cells were replaced with fresh culture medium. The cells were treated with 1 $\mu\text{g/ml}$ of LPS. The extracts were treated at 10, 25,

50 and 100 $\mu\text{g/ml}$. And cultured in a cell culture incubator for 24 hours. Thereafter, the supernatant that was obtained by centrifugation at 1,200 rpm for five minutes was measured with a PGE2 elisa kit (R&D Systems, USA) according to the manufacturer's instructions. The measurement result was obtained by multiplying the automatically calculated value by the dilution factor with the four-parameter logistic curve fit.

6. Inflammatory cytokines

Cells were plated at 1×10^5 cells/well in a 12-well plate and cultured for 24 hours. After cultivation, the cells were replaced with fresh culture medium. The cells were treated with 1 $\mu\text{g/ml}$ of LPS. And then cultured in a cell culture incubator for 24 hours. Then, centrifugation was carried out at 1,200 rpm for five minutes. Interleukin 1 β (IL-1 β), interleukin 6 (IL-6), and tumor necrosis factor α (TNF- α) were measured by the supernatant using a mouse cytokine immunoassay kit (Koma, Korea) according to the manufacturer's instructions, and the measured results were automatically calculated using a five-parameter logistic curve.

7. Antioxidative activity

For an antioxidant activity test of DPPH free-radical scavenging activity, 250 μM of DPPH was dissolved in ethanol. Woogakseungmatang extract was prepared at concentrations of 0, 10, 25, 50 and 100 $\mu\text{g/ml}$, mixed with 100 μl of DPPH solution. Absorbance was measured at 515 nm. Ascorbic acid (0, 10, 50, 100, 200, and 400 $\mu\text{g/ml}$) prepared at the same concentration was used as a positive control. The scavenging activity rate was calculated on the basis of the results without Woogakseungmatang extract.

DPPH free radical scavenging activity (%) =

$$\frac{\text{Absorbance of Control} - \text{Experimental}}{\text{Absorbance of Control}} \times 100$$

8. Statistical analysis

The experimental results were expressed as mean $\pm$ standard deviation (mean $\pm$ SD). One-way ANOVA analysis was used for each comparison and to verify statistical

significance (** $p < 0.01$; * $p < 0.05$).

Results

1. Cytotoxicity

The cell viability was 100 ± 0.49 , 99.46 ± 0.69 , 98.17 ± 0.57 , 97.55 ± 1.89 , 92.18 ± 2.97 , 85.39 ± 1.54 , and $76.11 \pm 2.81\%$ in the control group and at 10, 25, 50, 100, 150 and 200 $\mu\text{g/ml}$, respectively. *Woogakseungmatang* extract showed no cytotoxicity at the 10, 25, 50, and 100 $\mu\text{g/ml}$ concentrations, but decreased by about 15 and 24% at 150 and 200 $\mu\text{g/ml}$ compared with the control; thus, the 10, 25, 50, and 100 $\mu\text{g/ml}$ concentrations were selected in subsequent experiments (Fig. 1).

Fig. 1. This graph shows the cytotoxic effects of *Woogakseungmatang* extract in macrophage. There is no significant cytotoxicity up to 150 $\mu\text{g/ml}$.

2. NO production

The means of NO treated with LPS alone is 31.40 μM . The NO production was 100.00 ± 0.65 , 98.15 ± 1.95 , 93.79 ± 1.28 , 88.77 ± 2.18 , and $85.13 \pm 1.93\%$ in the control group and at 10, 25, 50, and 100 $\mu\text{g/ml}$, respectively. *Woogakseungmatang* extract showed approximately 85% NO production at 100 $\mu\text{g/ml}$ compared with the control; a significant difference was observed (Fig. 2).

3. PGE₂ production

The means of PGE₂ treated with LPS alone is 946.27 pg/ml. The PGE₂ production was 100 ± 0.42 , 99.51 ± 1.29 , 96.75 ± 2.68 , 89.16 ± 1.94 , and $82.64 \pm 2.54\%$ in the control group and at 10, 25, 50, and 100 $\mu\text{g/ml}$, respectively.

Woogakseungmatang extract showed a significant decrease in the amount of PGE₂ produced at 100 $\mu\text{g/ml}$ compared with the control (Fig. 2).

4. Inflammatory cytokines

Woogakseungmatang extracts were treated with cells, and their cytokine production was measured.

The means of IL-1 β treated with LPS alone is 231.62 pg/ml. The IL-1 β production was 100 ± 0.79 , 98.54 ± 1.28 , 96.41 ± 3.97 , 91.25 ± 2.19 , and $85.24 \pm 1.18\%$ in the control group and at 10, 25, 50, and 100 $\mu\text{g/ml}$, respectively.

The IL-1 β production was reduced by approximately 9% at 100 $\mu\text{g/ml}$ compared with the control. There was a

Fig. 2. LPS induced NO and PGE₂ production. RAW 264.7 cells were preincubated with 10, 25, 50 and 100 $\mu\text{g/ml}$ of *Woogakseungmatang*. The cells were treated with 1 $\mu\text{g/ml}$ of LPS to induce NO and PGE₂ production. The NO production was measured by Griess Reagent System, and the PGE₂ production was measured by ELISA as described in materials and methods. Data are represented as means $\pm$ SEM. Significantly different from control (#) or LPS alone (*); ### $p < 0.001$, * $p < 0.05$.

significant decrease at 100 $\mu\text{g/ml}$.

The means of IL-6 treated with LPS alone is 159.54 ng/ml. The IL-6 production was 100 ± 0.69 , 99.10 ± 1.96 , 95.31 ± 2.84 , 92.74 ± 1.48 , and $86.18\pm3.37\%$ in the control group and at 10, 25, 50, and 100 mg/ml, respectively. Woogakseungmatang extract reduced IL-6 production by approximately 12, 18, and 20% at the 25-, 50-, and 100- $\mu\text{g/ml}$ concentrations, respectively. There was a significant decrease at 100 $\mu\text{g/ml}$.

The means of TNF- α treated with LPS alone is 4395.59 pg/ml. The TNF- α production was 100 ± 0.78 , 98.52 ± 2.19 , 94.21 ± 3.46 , 90.77 ± 2.43 , and $84.20\pm3.94\%$ in the control group and at 10, 25, 50, and 100 $\mu\text{g/ml}$, respectively. The TNF- α production was also reduced by approximately 19 and 25% at 50 and 100 $\mu\text{g/ml}$, respectively. There was a significant decrease at 100 $\mu\text{g/ml}$ (Fig. 3).

5. Antioxidative activity

The DPPH radical scavenging activity was 18.26 ± 2.14 ,

23.87 ± 2.96 , 35.11 ± 3.73 , and $50.89\pm3.59\%$ compared with the control group at 10, 25, 50, and 100 $\mu\text{g/ml}$, respectively. The DPPH radical scavenging activity of ascorbic acid and Woogakseungmatang extract was measured. The ascorbic acid-treated group showed more than 100% activity at 50 $\mu\text{g/ml}$. Woogakseungmatang extract significantly increased the DPPH radical scavenging ability in a concentration-dependent manner. The criteria for the data is that the reagents have not been processed. Woogakseungmatang extract at 100 $\mu\text{g/ml}$ showed the highest DPPH radical scavenging ability (more than 50%) (Fig. 3).

Discussion

Seogak is rhinoceros horn. In Korean medicine, it is said to have the effect of lowering fever, reducing edema, and eliminating side effects; however, rhinoceros is an

Fig. 3. Inhibition of LPS-induced IL-1 β , IL-6, TNF- α and DPPH radical by Woogakseungmatang extract.

RAW 264.7 cells were preincubated with 10, 25, 50 and 100 $\mu\text{g/ml}$ of Woogakseungmatang extract. The cells were treated with 1 $\mu\text{g/ml}$ of LPS. The IL-1 β , IL-6 and TNF- α production was measured by ELISA as described in materials and methods. Data are represented as means $\pm$ SEM. Significantly different from control (#) or LPS alone (*); ###,*** $p<0.001$, * $p<0.05$.

internationally protected animal whose horns have limited use. Woogak has the same effect as Seogak because it has the effect of lowering and treating fever in Korean medicine. Seogak and Woogak have the same main components; therefore, Woogak is the most commonly used substitute for Seogak³⁾.

Macrophages protect the body from foreign substances, viruses, microorganisms, etc. when they invade. They are robust and have a longer lifespan than neutrophils; in addition, they protect against the treatment of waste products and microorganisms that neutrophils cannot digest; they are also known to produce inflammatory cytokines and antitumor effects and have been used in this study.

As a result of the cytotoxicity test for Woogakseungmatang, approximately 15% of cells were killed at the 200- μ g/ml concentration. Since this is a globally significant figure, the experiment proceeded to use 100 μ g/ml.

NO is an inflammatory factor and a major cause of various chronic inflammatory diseases. When inflammation occurs, macrophages are activated and produce NO. NO-activated leukocytes and macrophages help regenerate tissues after removing foreign matter; however, excess NO is known to cause cerebral infarction, degenerative neurological diseases, diabetes, and similar issues⁸⁾. Thus, Woogakseungmatang extract showed the effect of reducing NO by approximately 15% at 100 μ g/ml compared with the control, which is statistically significant.

The main symptoms of inflammation are pain, fever, redness, and swelling⁹⁾; PGE₂ modulates these symptoms and increases the vascular permeability to spread leukocytes to inflammation sites. It is considered an inflammatory mediator, but induces IgE secretion depending on the target cell and promotes interleukin-4 (IL-4) and interleukin-5 (IL-5) production¹⁰⁾. Woogakseungmatang extract showed the effect of reducing PGE₂ significantly by approximately 18% at 100 μ g/ml compared with the control.

In general, the inflammatory response of macrophages activated by LPS involves inflammatory mediators, such as pro-inflammatory cytokines, NO, PGE₂, LTB₄, iNOS, and COX-2¹¹⁾. Representative inflammatory cytokines involved in

the development of inflammation are IL-1 β , IL-6, and TNF- α . Among these, IL-1 β causes an inflammatory reaction, is involved in fever, and activates lymphocytes and neutrophils. IL-6 promotes the production of acute-phase protein (CRP) and develops chronic stages of inflammatory diseases, including allergic diseases. In addition, TNF- α activates leukocytes by causing an inflammatory reaction. However, excess cytokine causes thrombosis and tissue damage¹²⁾. In this study, Woogakseungmatang extract significantly decreased IL-1 β production at the 100- μ g/ml concentration, IL-6 production was significantly decreased at 10 and 100 μ g/ml, and TNF- α was significantly reduced at 100 μ g/ml. These results show the potential of Woogakseungmatang extract to be used to prevent and treat various inflammatory diseases through the effective reduction of inflammatory cytokines¹³⁾.

Studies on the relationship between the cause of disease and oxidative byproducts by reactive oxygen species are underway¹⁴⁾. Active oxygen is known to be harmful to proteins, biological membranes, and DNA. Therefore, studies on antioxidants have been actively conducted recently¹⁵⁾. The DPPH free radical scavenging capability showed a tendency to increase in a concentration-dependent manner after Woogakseungmatang extract treatment. At the concentration of 100 μ g/ml, it showed more than 50% scavenging ability.

Woogakseungmatang extract inhibits the inflammatory process by significantly reducing cytokine IL-6 and TNF- α production. Woogakseungmatang extracts should be studied clinically and experimentally.

Conclusion

This paper investigates the anti-inflammatory effect of Woogakseungmatang extract using RAW 264.7 cells to measure the amounts of MTT, NO, and PGE₂ and cytokines IL-1 β , IL-6, and TNF- α . Thus, the following conclusions were obtained:

1. There was no significant change in cell survival up to 100 μ g/ml for Woogakseungmatang extract; cell viability was 76% at 200 μ g/ml.

2. The amount of NO produced was significantly decreased by 85% at 100 μ g/ml compared with the control.

3. The amount of PGE₂ produced was significantly reduced by 82% at 100 μ g/ml compared with the control.

4. IL-1 β , IL-6, and TNF- α were significantly reduced at 100 μ g/ml compared with the control group.

5. The DPPH free radical scavenging capability was more than 50% at 100 μ g/ml.

According to this study, the Woogakseungmatang showed only a slight anti-inflammatory effect at 100 μ g/ml the highest concentration that does not show cytotoxicity. In addition, it was difficult to confirm the concentration-dependent anti-inflammatory effect of the Woogakseungmatang. Therefore, this study means to confirm the potential anti-inflammatory effects of Woogakseungmatang. A variety of anti-inflammatory studies of Woogakseungmatang are needed.

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국문초록

목적 : 우각승마탕은 한의학에서 안면마비 등 안면부의 통증 및 마비를 동반하는 질환에 주로 사용하는 처방이다. 본 연구의 목적은 LPS로 염증 반응을 유도한 대식세포에서 우각승마탕의 항염증 및 항산화 효과를 알아 보는 것이다. **방법 :** 동의보감 처방에 기반한 우각승마탕을 열수 추출한 후 동결건조 하였다. MTT 방법을 이용하여 세포 독성을 평가하였고, 우각승마탕 추출물을 처리한 RAW 세포에서 NO 생산 및 PGE₂ 생성량을 조사 하였다. 또한 염증과 관련된 사이토카인(IL-1 β , IL-6, and TNF- α)의 생성량 변화를 조사 하였다. 우각승마탕의 항산화 효능은 DPPH 법으로 측정 하였다. **결과 :** 우각승마탕 추출물을 처리 한 대식세포는 100 μ g/ml 농도에서 92.18 $\pm$ 2.97%의 세포 생존율을 보였다. 150과 200 μ g/ml 농도에서 세포생존율은 각각 85.39 $\pm$ 1.54 and 76.11 $\pm$ 2.81%로 통계적으로 유의한 감소가 있었다. 100 μ g/ml 농도에서 NO 생성량은 85.13 $\pm$ 1.93% 였으며, 대조군과 비교하여 유의한 감소가 나타났다. PGE₂ 생성량은 100 μ g/ml 농도에서 82.64 $\pm$ 2.54% 이었고, 통계적으로 유의한 감소가 있었다. IL-1 β , IL-6, TNF- α 는 100 μ g/ml에서 각각 85.24 $\pm$ 1.18, 86.18 $\pm$ 3.37, 84.20 $\pm$ 3.94%였으며, 대조군과 비교하여 통계적으로 유의한 감소를 나타내었다. DPPH free radical 소거능은 100 μ g/ml에서 50.89 $\pm$ 3.59%를 나타내었다. **결론 :** 본 연구의 결과로 미루어 볼 때 우각승마탕은 세포독성을 보이지 않는 최고 농도인 100 μ g/ml에서 미약한 항염증 효과를 나타내었고 농도의존적 항염증 효과를 확인되지 않았다. 따라서 본 연구는 우각승마탕의 잠재적 항염증 효과를 확인하는 의미가 있었다. 이 연구를 바탕으로 우각승마탕의 안전하고 효과적인 임상적 사용을 위하여 체계적이고 다양한 연구가 수행되어야 할 것이다.

키워드 : 우각승마탕, 서각승마탕, 항염, 항산화, 대식세포, 한의학