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Inhibitory effect of *Smilacis Glabrae Rhizoma* on nitric oxide production in the macrophage cell line RAW 264.7

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土茯笭이 RAW264.7 대식세포주에서 산화질소 억제에 미치는 영향

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Abstract

Objectives : 본 연구의 목적은 제습, 해독, 통리관절등의 효능이 있는 토복령이 RAW264.7 대식세포주에서 lipopolysaccharide(LPS)로 처치하여 생성되는 nitric oxide(NO)와 prostaglandin E_2(PGE_2)에 억제작용이 있는지 관찰하고자 하는 것이다.

Methods : 토복령이 RAW264.7 대식세포주에 세포독성이 유무를 관찰하기 위하여 농도별 MTT assay를 수행하여 세포생존율을 측정하였다. 또한 LPS로 염증유발된 RAW264.7 대식세포주에서 토복령의 농도별 처치가 NO 및 PGE_2 생성억제에 미치는 영향을 관찰하고자 NO 및 PGE_2 assay를 수행하였다.

Results : 토복령의 농도별 처치가 RAW264.7 대식세포주에서 세포독성을 유발하는지 MTT assay로 측정한 결과 세포독성은 관찰되지 않았으며, 토복령은 LPS처치로 인하여 증가된 NO 및 PGE_2 생성을 통계학적으로 유의하게 감소시킴을 관찰하였다.

Conclusions : 본 연구를 통하여 토복령은 RAW264.7 대식세포주에서 LPS로 유도된NO 및 PGE_2 생성을 효과적으로 억제시킴으로써 추후 염증질환의 치료제로서 가능성을 확인하였다.

Key words : 대식세포주, 산화질소, 토복령, 프로스타글란딘

Ⅰ. Introduction

Smilacis glabrae rhizoma (SGR) is the rhizome of the Liliaceae plant, Smilax glabra Roxb. In traditional Oriental medicine, SGR has been considered to be effective in detoxicating, relieving dampness, and easing joint movement, and has been used clinically for a long time to prevent leptospirosis, and to treat syphilis, acute bacterial dysentery, acute and chronic nephritis, etc[1)]. Previous studies have demonstrated that the aqueous extract from SGR showed a remarkable inhibition on the delayed-type hypersensitivity (DTH)

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reactions induced by picryl chloride or sheep red blood cells mainly through affecting the effector phase of DTH with an anti-inflammatory action but without inhibiting humoral immune response[2]. Such selectivity of SGR extract for inhibiting cellular immune response (CIR) is different from that of glucocorticoids and immunosuppressing agents, and may be advantageous to the treatment of CIR-mediated inflammation[3].

One of the mediators causing inflammation in several organ is nitric oxide (NO). The excessive production of this free radical is pathogenic to the host tissue, since NO can bind with other superoxide radicals which directly damages the function of normal cells[4]. NO is produced by the oxidation of L-arginine catalyzed by NO synthase (NOS). Inducible NOS (iNOS) is particularly well known to be involved in the overproduction of NO in cells and it is important pharmacological target in inflammation[5].

Prostaglandin E_2 (PGE_2) and tumor necrosis factor-alpha (TNF-α) are also inflammatory mediators that involved in various pathophysiological processes including increased vascular permeability, vascular dilation and neutrophil chemotaxis. PGE_2 is derived from the catalyzation of arachidonic acid by cyclooxygenase-2 enzyme (COX-2) pathway. The cells that produce large amount of PGE_2 are monocytes and macrophages, whereas those for TNF-α and IL-4 are macrophages, mast cells and basophils[6]. Thus, searching for inflammagory inhibitors from natural sources is an alternative approach in development of anti-inflammatory drugs[5].

Macrophage plays an important role in the host defense mechanism, when activated it inhibited the growth of a wide variety of tumor cells and microorganisms. The synthesis of NO by activated macrophages is an important cytotoxic/cytostatic mechanism of non-specific immunity[7]. Original evidence of tumor cell cytostasis and cytotoxity was found in macrophage-tumor cell co-cultured in which cytokine and/or lipopolysaccharides (LPS)-stimulated macrophages inhibited metabolic functioning of co-cultured tumor cells[8]. The expression of NO has been linked with DNA damage, thus stimulating the expression of p53 and ultimately leading to apoptosis[9]. Thus, NO-mediated tumoricidal effects, similar to its anti-microbial effects, require antigen recognition but result in antigen-nonspecific killing. For the induction of iNOS gene expression in macrophage, IFN-γ is required as a priming signal before it can subsequently be triggered by a second signal, such as, bacterial LPS or pro-inflammatory cytokines[10].

Therefore, in the present study we investigated the inhibitory activity of SGR and the extract against NO and PGE_2 releases using RAW264.7 macrophage cells.

II. Materials and Methods

1. Cell culture

Rat peritoneal cells were cultured with 1640 medium (final LPS content < 10 pg/ml) in 24-well plated (2 x 10^6 macrophages per well in a volume of 1 ml)(Falcon) at 37℃ in 5% CO_2 /95% air and enriched for macrophages by 2-3 h adherence steps as described. Then, they were incubated in the absence or presence of LPS for 6 h for NO and PGE_2 assay, respectively. Each assay was carried out in triplicate.

2. Preparation of SGR extracts

SGR was purchased from Semyung Oriental Hospital and identified as Smilacis glabra Roxb. by Prof. Leem (Department of Herbology, College of Oriental Medicine, Semyung University). The aqueous extract from SGR was made by a common method. Briefly, the SGR material was extracted twice with 10 times distilled water at 80℃ for 1 h. Then, the supernatant after centrifugation at 200 g was filtered and lyophilized to obtain a powder with 10% of yield. The dosage of SGR extract used in this study was indicated as this powder. The dried extract was dissolved in phosphate-buffered saline (PBS) and filtered with 0.22-㎛ syringe filter.

3. MTT assay

To test the viability of cells, MTT colorimetric assay was performed as described. Briefly, macrophages were incubated for 6 h after stimulation with the absence or presence of SGR. After addition of MTT solution, the cells were incubated at 37℃ for 4 h. The crystallized MTT was dissolved and measured the absorbance at 540 nm.

4. Measurement of NO

In the presence of H_2O, NO is rapidly converted to nitrite and nitrate. Total production of NO therefore may be determined by assaying for nitrite. To assay nitrite, the cell seeded in 96-well plates with 1 x 10^5 cells/well and allowed to adhere for 1 h at 37℃ in a humidified atmosphere containing 5% CO_2. After that the medium was replaced with a fresh medium containing 100 ug/ml of LPS together with the test samples at various concentations and was then incubated for 48 h. NO production was determined by

measuring the accumulation of nitrite in the culture supernatant using the Griess reagent. The OD values of the samples were then read at 540 nm.

5. Measurement of PGE_2 production

The cell seeded in 96-well plates with 1 x 10^5 cells/well and allowed to adhere for 1 h at 37℃ in a humidified atmosphere containing 5% CO_2. After that the medium was replaced with a fresh medium containing 100 ug/ml of LPS together with the test samples at various concentations and was then incubated for 48 h. The supernatant was transferred into 96-well ELISA plate and then PGE_2 concentrations were determined using commercial ELISA kits. The test sample was dissolved in DMSO, and the solution was added to RPMI. The inhibition on PGE_2 releases was calculated.

6. Statistical analysis

Results were expressed as the mean±SEM. Differences between groups were assessed by the student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

III. Results

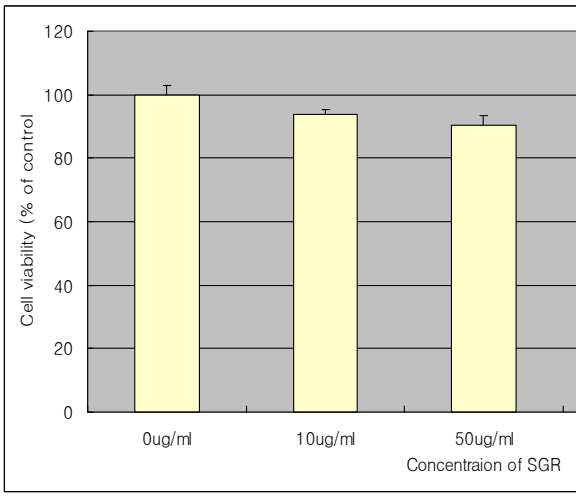

Fig. 1. Influence of aqueous extract of SGR on the cell viability was determined using the MTT assay. Each v

1. MTT assay

As shown in Fig. 1, the viabilities of cells incubated with SGR at concentration of 0, 10, 50, 100 and 200 ㎍/㎖ were 100.00 ± 3.01, 93.92 ± 1.51, 90.54 ± 2.78, 96.23 ± 3.58, and 94.11 ± 1.80 of the control value, respectively. The MTT assay revealed that SGR exerted no significant cytotoxicity in the macrophage.

2. Effect of SGR on LPS-induced NO production in RAW264.7 macrophage cells

When the cells incubated with LPS, the amount of NO production was increased significantly from 3.58 ± 0.21 uM to 19.70 ± 0.24 uM. However, SGR decreased significantly to 11.80 ± 0.12 uM, 10.34 ± 0.08 uM and 9.52 ± 0.16 uM at dose range 10, 50 and 100 ㎍/㎖ (Fig.2).

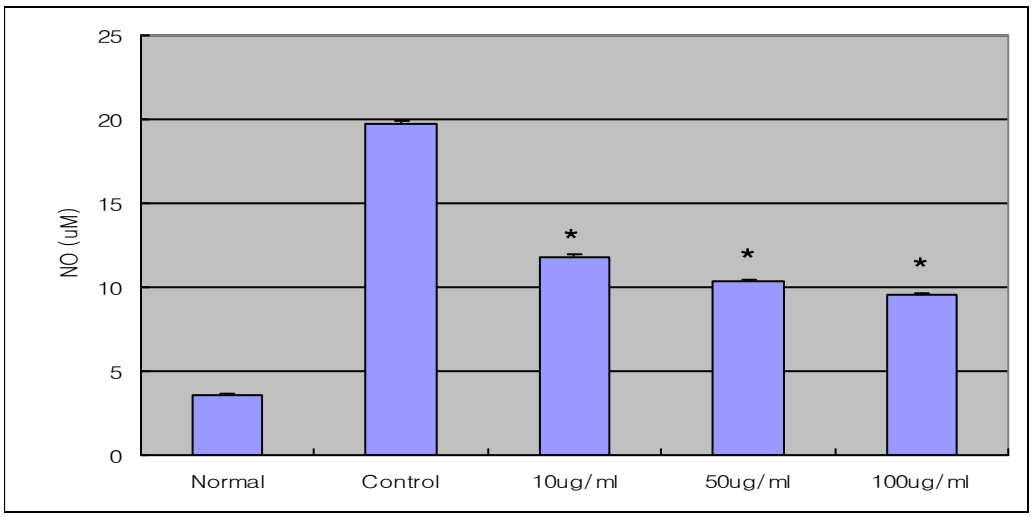

Fig. 2. Effects of aqueous extract of SGR on LPS-induced NO production in RAW264.7 macrophage cells. RAW264.7 macrophage cells were incubated with different concentrations of SGR extract in the presence of LPS. Three independent experiments were performed, and data represent the mean ± SEM. *represents $p<0.05$ compared to the LPS treated control group.

3. Effect of SGR on LPS-induced PGE2 production in RAW264.7 macrophage cells

When the cells incubated with LPS, the amount of PGE2 production was increased significantly from 35.78 ± 7.74 pg/well to 50.31 ± 7.21 pg/well. However, SGR decreased significantly to 33.43 ± 1.80 pg/well at dose range 100 ㎍/㎖ (Fig.3).

IV. Discussion

SGR used in Korean traditional medicine for treatment of chronic nephritis and arthritis, was investigated for anti-inflammatory activity using RAW264.7 macrophage cell line. First, SGR exerted no

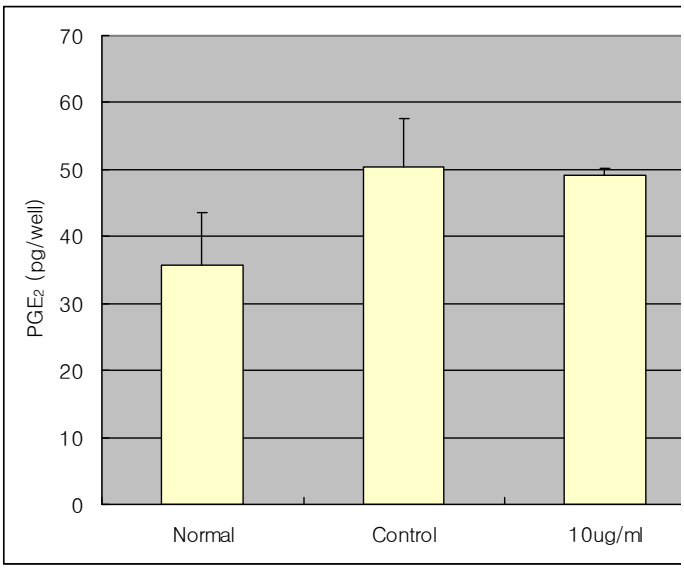

Fig. 3. Effects of aqueous extract of SGR on LPS-induced PG cells. RAW264.7 macrophage cells were incubated with different concen Three independent experiments were performed, and data represent the the LPS treated control group.

significant cytotoxicity in the macrophage using by MTT cytotoxic assay.

Additionally, our investigation found that SGR showed a significant inhibition of the NO and PGE_2 production. These results reflected that SGR exhibit its inhibitory activity on LPS-induced RAW264.7 macrophage.

The major finding of this study is that SGR significantly inhibited NO production and PGE_2 expression. Inflammation is the first response of the immune system to infection or irritation. During the inflammatory process, large amounts of the pro-inflammatory mediators NO and PGE_2 are generated by the inducible isoforms of iNOS and COX-2[11]. thus, inhibitors of the production of these inflammatory mediators have been considered as a candidate for anti-inflammatory drugs.

NO is involved in various pathophysiological processes including inflammation and septic shock[12,13]. At adequate concentrations, NO can generate or modify intracellular signals, thereby affecting the function of immune cells, as well as tumor cells and resident cells of different tissues and organs. However, its uncontrolled release can cause inflammatory destruction of target tissue during an infection[14-16]. The modulation of iNOS-mediated NO release is one of the major contributing factors during the inflammatory process[17]. Therefore, agents with the ability to inhibit iNOS expression are potentially beneficial in the treatment of conditions associated with an overproduction of NO, including septic shock and inflammation[18].

PGE_2 is another proinflammatory mediator involved in inflammatory responses and it is generated by the sequential metabolism of arachidonic acid by COX[19]. COX exits in two isoforms: COX-1 and COX-2. COX-1 is a constitutively expressed enzyme with general housekeeping functions. COX-2 is an inducible isoform of COX, and its most important role is in inflammation[20,21], and it is responsible for proinflammatory PGE_2 production. Similar to iNOS inhibition, we found that SGR reduced the production of PGE_2 in RAW264.7 cells.

In summary, the findings of the present study suggest that SGR is an effective inhibitor of LPS-induced NO and PGE_2 production in murine RAW264.7 macrophages. And, it offers a possible approach to the treatment of severe inflammatory disease.

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