



ORIGINAL ARTICLE

Effect of Red Ginseng Oil on Cultured Sebocytes and Outer Root Sheath Cells after Treatment with Lipopolysaccharide

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Background: Ginseng has been known in Korea as a health-supportive herbal medicine from time immemorial. Essential oil isolated from fresh ginseng has been shown to display antibacterial and anti-inflammatory activities. **Objective:** The effects of red ginseng oil (RGO) on the lipopolysaccharide (LPS)-treated sebocytes and outer root sheath (ORS) cells were studied. **Methods:** The cultured cells were treated with either 0.1% dimethyl sulfoxide, 5 μ g/ml LPS, 50 μ g/ml RGO, or 5 μ g/ml LPS plus 50 μ g/ml RGO for 6 and 24 hours. RT-PCR, real-time PCR, enzyme-linked immunosorbent assay, western blot, and immunofluorescence staining were performed for the analysis of inflammatory cytokine. **Results:** RGO showed the increased gene and protein expression of inflammatory cytokines, including interleukin (IL)-1 β , IL-6, IL-8, and tumor necrosis factor- α in the LPS-treated sebocytes and ORS cells. RGO also showed the increased protein expression of p-c-jun and p-JNK in the LPS-treated sebocytes and ORS cells. Gene expression of TLR2 was increased in LPS-treated sebocytes following treatment with RGO. Additionally, RGO resulted in an increased expression of LL-37 in the LPS-treated sebocytes and ORS cells. Moreover, it remarkably increased the production of sebum in LPS-treated

sebocytes. **Conclusion:** RGO might be among the aggravating factors of acne vulgaris. It would be better to stop taking red ginseng in patients with inflammatory acne. (**Ann Dermatol 33(3) 245 ~ 253, 2021**)

-Keywords-

Acne vulgaris, Outer root sheath cells, Red ginseng oil, Sebocytes

INTRODUCTION

Acne is a very common inflammatory disorder happening in the face, chest, and back during the adolescent period. Its development is correlated with excessive sebum production, *Cutibacterium acnes* proliferation, follicular hyperkeratosis, and inflammation. Also, it can be aggravated by stressful conditions, hormonal imbalances, and nutritional factors. It is handled by a variety of therapeutic options, including topical ointments, oral medications, and surgical devices. Nonetheless, other therapeutic options are still needed for the complete and safe treatment of acne. Complementary and alternative medicines are among the therapeutic options for acne vulgaris.

In Eastern Asia, ginseng is known as a noble, miraculous herbal medicine with a potential to treat several health problems¹. Thus, ginseng has been utilized to maintain immune homeostasis and enhance resistance to disease or microbial attacks via immune modulation^{2,3}. The advantageous effects of ginseng come from ginsenosides, which are the chief ingredients of ginseng. Recently, the pharmacological effects of non-ginsenosides have been reported. So, the biological impacts of essential oils extracted from different herbs and plants have gained increasing atten-

Received August 3, 2020, Revised September 23, 2020, Accepted for publication October 19, 2020

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tion⁴. Essential oil isolated from fresh ginseng, red ginseng oil (RGO), has been shown to display antibacterial and anti-inflammatory activities⁵.

This study was performed to study the impact of RGO on the acne-related cultured cells. Cultured sebocytes and outer root sheath (ORS) cells of hair were utilized for the *in vitro* test.

MATERIALS AND METHODS

Sebocyte and outer root sheath cell culture

Sebaceous glands were isolated from occiput hair follicles and transferred to Biocoat collagen type I-coated culture dishes (CORNING, Kennebunk, ME, USA). Informed written consent was acquired. The Medical Ethical Committee of the Kyungpook National University Hospital (Daegu, Korea) approved all of the described studies (IRB no. KNU 2018-0155). The explants were maintained in Dulbecco's Modified Eagle Medium (DMEM; Hyclone Laboratories, Logan, UT, USA) at 37°C in a humidified atmosphere of 5% CO₂. Explants were left for 4 days, and the medium was modified to EpiLife (MEPI500CA; Gibco BRL, Grand Island, NY, USA). The cells were harvested with 0.25% trypsin/10-mM EDTA in Hank's Balanced Salt Solution (HBSS) and then sub-cultured.

ORS cells were isolated from hair shafts of hair follicles. Trimmed hair follicles were immersed in DMEM, which is supplemented with 20% fetal bovine serum in Biocoat collagen type I-coated culture dishes (CORNING). After culturing for 3 days, the medium was replaced with EpiLife. Cells from the second passage were utilized in the experiments.

Red ginseng oil preparation

The supercritical CO₂ extract preparation was conducted in accordance with the earlier studies^{5,6}. The RGO was dissolved in dimethyl sulfoxide (DMSO) and diluted using sterile phosphate-buffered saline solution (PBS; pH 7.4) with Tween 20 (0.5% v/v for easy diffusion) as described in earlier studies^{4,7}. RGO was kindly provided by the Research Institute of Technology, Korea Ginseng Corporation (Daejeon, Korea).

MTT assay

The optimal concentration of RGO in sebocytes and ORS cells cultures was determined using MTT assay. Primary sebocytes and ORS cells were plated in 96-well, collagen-coated plates (5,000 cells per well; CORNING) for 24 hours. On the day following seeding, sebocytes and ORS cells were incubated for 3 days without a supplement medium in the absence or presence of RGO. MTT solution

(3-[4,5]dimethylthiazol-2,5-diphenyltetrazolium bromide) was then added at 70 μg per well for 3 hours. The formazan product was dissolved with DMSO, and optical density was calculated at 570 nm. It showed that 50 μg/ml RGO caused no changes in cell viability, and 50 μg/ml RGO was used *in vitro* test.

RT-PCR analysis and real-time PCR

Cells were treated with 0.1% DMSO, 5 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO, USA), 50 μg/ml RGO, or 5 μg/ml LPS plus 50 μg/ml RGO for 6 and 24 hours. The dose of RGO was determined by MTT assay. Total RNA was acquired using a RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized from 3 μg of total RNA with the use of a cDNA synthesis kit containing ImProm-ITM reverse transcriptase and oligo-dT primers, in accordance with the manufacturer's instructions (Promega, Madison, WI, USA). One microliter of cDNA was amplified with each of the forward and reverse primers. For detecting human LL-37, 30 cycles (45 seconds at 94°C, 45 seconds at 60°C, and 45 seconds at 72°C) of amplification were conducted (forward primer 5'-GGGTAGGGCACACTAGGA-3', reverse primer 5'-GGACAGTGACCCTCAACCAG-3'). For human TLR2, 28 cycles (45 seconds at 94°C, 45 seconds at 57°C, and 45 seconds at 72°C) of amplification was conducted (forward primer 5'-GGCCAGCAAATTACCTGTGT-3', reverse primer 5'-TTCTCCACCCAGTAGGCATC-3'). For human TLR4, 28 cycles (45 seconds at 94°C, 45 seconds at 57°C, and 45 seconds at 72°C) of amplification was conducted (forward primer 5'-CCATGTTTCATTGTGGCACTC-3', reverse primer 5'-TCCCTTCCTCCTTTCCCTA-3'). For human β-actin, 23 cycles (45 seconds at 94°C, 45 seconds at 58°C, and 45 seconds at 72°C) of amplification were conducted (forward primer 5'-GGGAAATCGTGCGTGACATT-3', reverse primer 5'-GGAGTTGAAGGTA GTTTCGT-3'). PCR products were separated by electrophoresis on a 1% agarose gel and visualized under ultraviolet light.

The real-time PCR was conducted using Step one Plus real-time PCR Assay (Applied Biosystems, Foster City, CA, USA). All reactions were conducted by Power SYBR Green premix (Applied Biosystems) using 50 ng cDNA and 10 pM interleukin (IL)-1β (Qiagen, Hs-IL1B-SG), IL-6 (Qiagen, Hs-IL6-SG), IL-8 (Qiagen, Hs-IL8-SG), and tumor necrosis factor (TNF)-α specific oligonucleotide primers (Qiagen, Hs-TNF-SG). Amplification was conducted under the following cycling conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The differences between samples and controls were measured using the Step one Plus real-time PCR analysis software (Applied Biosystems).

ELISA

Analysis of IL-1 β , IL-6, IL-8, and TNF- α (R&D Systems, Minneapolis, CA, USA) protein expression using enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer's instructions. To calculate the protein levels in the conditioned medium of sebocytes and ORS cells from passage 2, they were plated overnight at a density of 30,000 cells/24-well culture dish and were washed thrice with PBS. To study the protein induction in cells in response to the RGO, the cells were treated with varying concentrations of RGO in serum-free medium for 6 or 24 hours, and protein concentrations in the conditioned medium were measured.

Cell death ELISA

This assay was conducted using a cell death ELISA kit (Roche, Mannheim, Germany), following the manufacturer's instructions. Briefly, following the treatment with 0.1% DMSO, 5 μ g/ml LPS, 50 μ g/ml RGO, or 5 μ g/ml LPS plus 50 μ g/ml RGO for 24 hours, cytoplasmic extracts were prepared from 20,000 sebocytes and ORS cells of passage 2, and DNA-histone complexes were calculated by reading optical density at 405 nm with the use of a microplate reader.

Western blot analysis

Cells were treated with 0.1% DMSO, 5 μ g/ml LPS, or 5 μ g/ml LPS plus 50 μ g/ml RGO for 6 hours. Proteins (10 μ g/lane) were separated with the use of 10% SDS-PAGE

and transferred to nitrocellulose membranes. The membranes were then blocked with 5% skim milk in PBS for 1 hour. They were probed with rabbit polyclonal antibodies against p-c-jun (1:1,000 dilution; Cell Signaling, Beverly, MA, USA), p-JNK (1:1,000 dilution; Cell Signaling), or p-iKB (1:1,000 dilution; Cell Signaling). Horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G (Jackson ImmunoResearch, Baltimore, PA, USA) was utilized as the secondary antibody at a 1:7,000 dilution. The bands were visualized using SuperSignal West Femto (Thermo Scientific, Rackford, IL, USA). The membranes were also probed with mouse monoclonal antibody against actin (1:5,000 dilution; Chemicon, Temecula, CA, USA) to assess the quantity and integrity of the protein samples.

Quantification of lipid production

The supernatant of RGO-treated sebocytes and ORS cells for 24 hours was collected in a clean 1.5 ml e-tube and stored at -20°C until required. The TG-S reaction kit (Asan Pharm. Co., Seoul, Korea) was utilized to detect neutral lipids, according to the manufacturer's protocol.

Immunofluorescence staining

For immunofluorescence staining of p-c-jun, cells were cultured in the EpiLife medium with 0.1% DMSO, 5 μ g/ml LPS, or 5 μ g/ml LPS plus 50 μ g/ml RGO for 6 hours. Fixation and blocking procedures were conducted as shown earlier. Cells were incubated with rabbit polyclonal p-c-jun antibody (1:100 dilution; Cell Signaling) at 4°C overnight, washed thrice with PBS, and incubated with Alexa Fluor

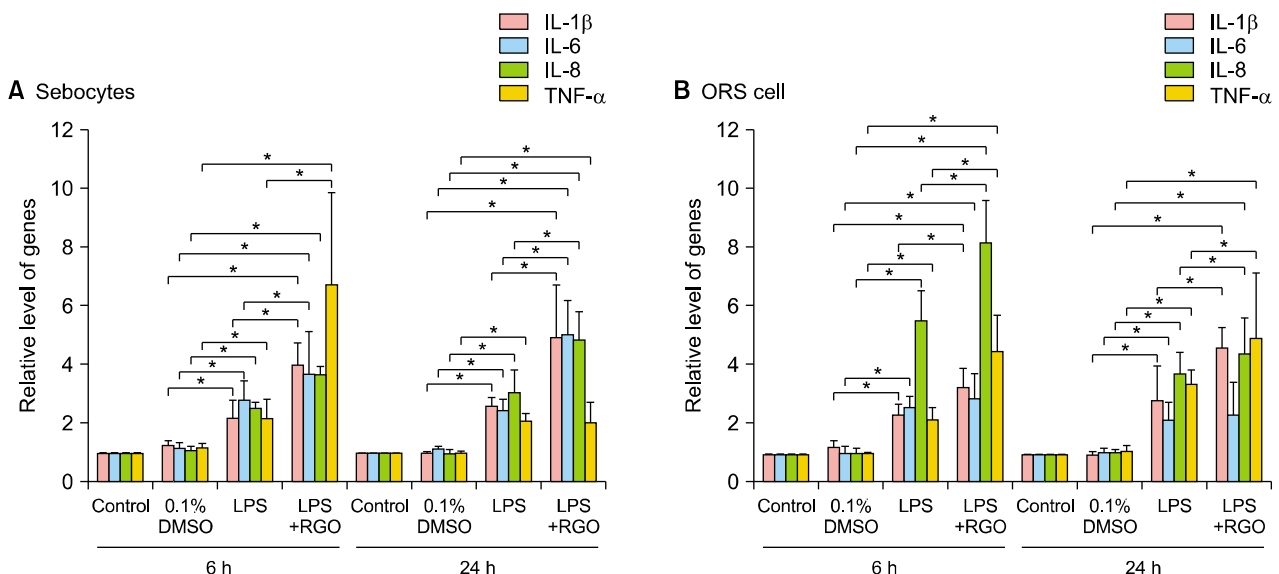


Fig. 1. RGO indicated an increased gene expression of inflammatory cytokines in the LPS-treated sebocytes (A) and ORS cells (B). Values are presented as mean \pm standard deviation from three independent experiments ($*p < 0.05$). IL: interleukin, TNF: tumor necrosis factor, DMSO: dimethyl sulfoxide, LPS: lipopolysaccharide, RGO: red ginseng oil, ORS: outer root sheath.

488-labeled donkey anti-rabbit secondary antibody (Molecular Probes, Eugene, OR, USA). Cells were then washed with PBS and counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes.

Statistical analysis

Data were expressed as mean ± standard deviation. ANOVA was utilized for statistical analysis of the data. A $p < 0.05$ was considered to be statistically significant.

RESULTS

Sebocytes were different from ORS cells in the expression of cytokeratin

In immunocytochemistry, sebocytes were positive for cytokeratin (CK) 1-3, CK 8, CK 15, CK 17, and CK 19 (Supple-

mentary Fig. 1A), but ORS cells were positive only for CK 1-3 and CK 8 (Supplementary Fig. 1B). Sebocytes were different from ORS cells in the expression of CK in immunocytochemistry.

Red ginseng oil indicated the increased expression of inflammatory cytokines in the LPS-treated sebocytes and ORS cells

In real-time PCR, the expression of IL-1 β was increased in the LPS-treated sebocytes and ORS cells 6 and 24 hours following the treatment with RGO ($p < 0.05$) (Fig. 1). The expression of IL-6 was increased in the LPS-treated sebocytes 6 and 24 hours following the treatment with RGO ($p < 0.05$) (Fig. 1A). The expression of IL-8 was increased in LPS-treated sebocytes 24 hours following the treatment with RGO ($p < 0.05$) (Fig. 1A). The expression of IL-8 was

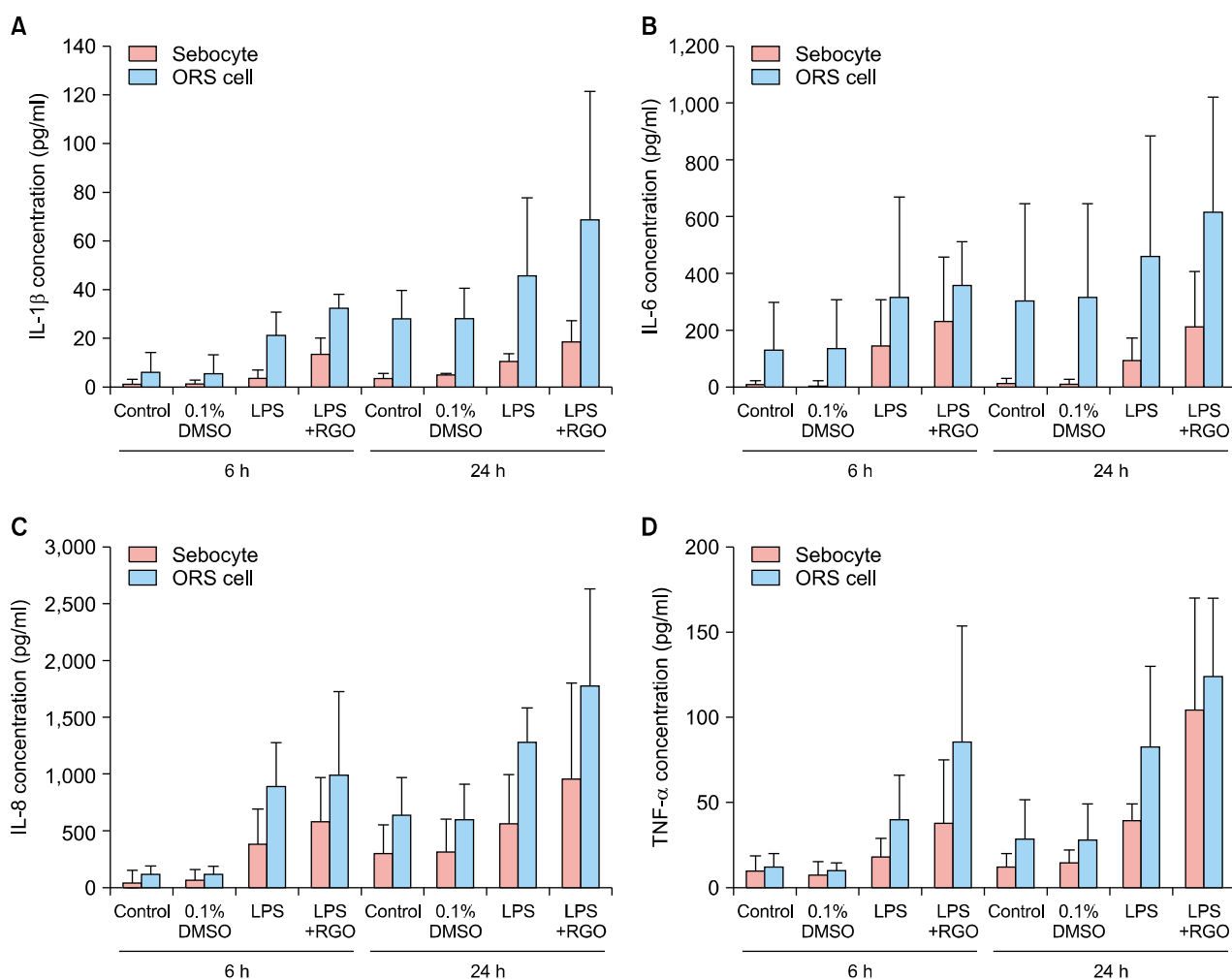


Fig. 2. RGO indicated an increased protein expression of inflammatory cytokines, such as IL-1 β (A), IL-6 (B), IL-8 (C), and TNF- α (D), in the LPS-treated sebocytes and ORS cells. Values are presented as mean ± standard deviation from three independent experiments. IL: interleukin, TNF: tumor necrosis factor, DMSO: dimethyl sulfoxide, LPS: lipopolysaccharide, RGO: red ginseng oil, ORS: outer root sheath.

also increased in LPS-treated ORS cells 6 and 24 hours following the treatment with RGO ($p < 0.05$) (Fig. 1B). The expression of TNF- α was also increased in LPS-treated sebocytes 6 hours following the treatment with RGO and in LPS-treated ORS cells 6 and 24 hours following the treatment with RGO ($p < 0.05$) (Fig. 1).

In ELISA, protein expression levels of IL-1 β , IL-6, IL-8, and TNF- α were increased in the LPS-treated sebocytes and ORS cells 6 and 24 hours following the treatment with RGO (Fig. 2). Nonetheless, protein expression levels of IL-1 β , IL-6, IL-8, and TNF- α were not increased in the LPS-not-treated sebocytes and ORS cells 24 hours following the treatment with RGO (Fig. 3).

Cell death ELISA indicated that apoptosis increased in the LPS-treated sebocytes and ORS cells, but not in the RGO-treated sebocytes and ORS cells (Supplementary Fig. 2A).

Also, it was shown that apoptosis increased in the LPS-treated sebocytes and ORS cells 24 hours following the treatment of RGO (Supplementary Fig. 2B).

Red ginseng oil indicated an increased expression of inflammatory cytokines via the pathway of c-jun in the LPS-treated sebocytes and ORS cells

In western blot analysis, the expression of p-c-jun and p-JNK was increased in LPS-treated sebocytes and ORS cells 6 hours following the treatment with RGO ($p < 0.05$) (Fig. 4A, B). In immunofluorescence, the expression of p-c-jun was increased in LPS-treated sebocytes and ORS cells 6 hours following the treatment with RGO (Fig. 4C). In RT-PCR, the mRNA expression of TLR2 was increased in LPS-treated sebocytes 24 hours following the treatment with RGO ($p < 0.05$) (Fig. 5) as well.

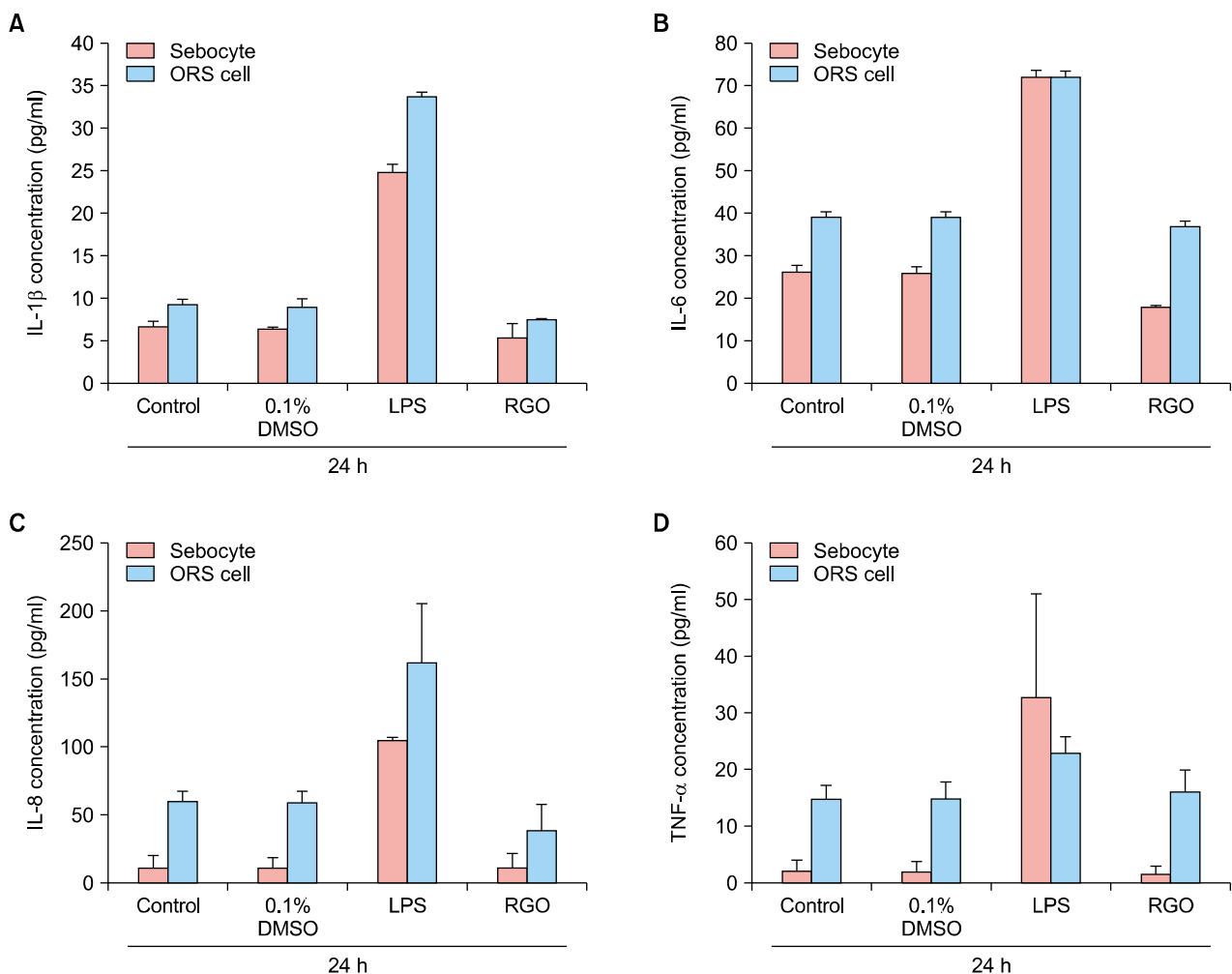


Fig. 3. RGO did not indicate an increase in the protein expression levels of IL-1 β (A), IL-6 (B), IL-8 (C), and TNF- α (D) in the LPS-not-treated sebocytes and ORS cells. Values are presented as mean \pm standard deviation from three independent experiments. IL: interleukin, TNF: tumor necrosis factor, DMSO: dimethyl sulfoxide, LPS: lipopolysaccharide, RGO: red ginseng oil, ORS: outer root sheath.

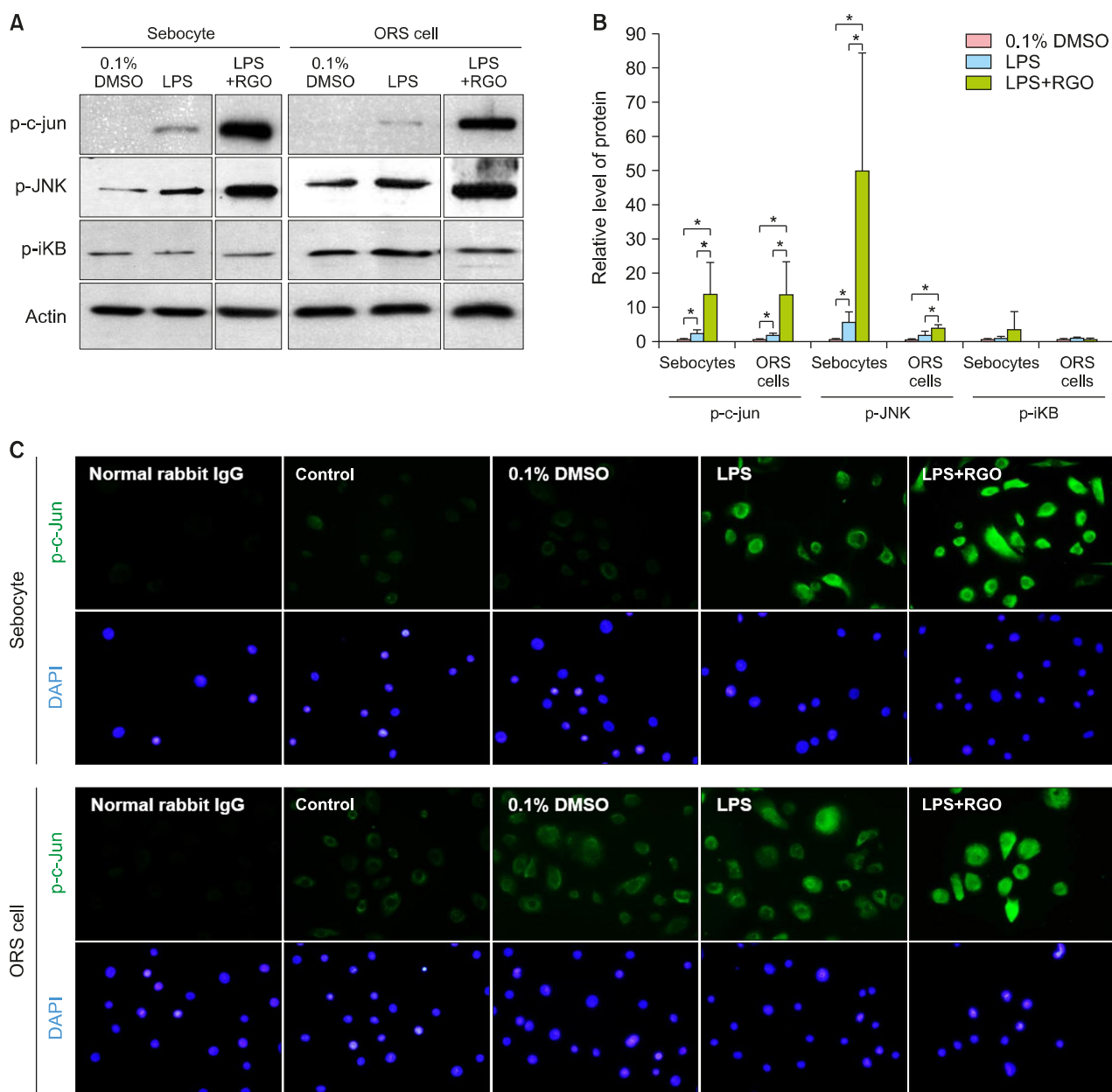


Fig. 4. (A, B) RGO indicated an increased protein expression of p-c-jun and p-JNK in the LPS-treated sebocytes and ORS cells in western blot analysis. Values are presented as mean±standard deviation from three independent experiments (* $p < 0.05$). (C) RGO indicated an increased protein expression of inflammatory cytokines via the pathway of p-c-jun in the LPS-treated sebocytes and ORS cells in immunofluorescence staining. ORS: outer root sheath, DMSO: dimethyl sulfoxide, LPS: lipopolysaccharide, RGO: red ginseng oil.

Red ginseng oil increased the expression of LL-37 in the LPS-treated sebocytes and ORS cells and the production of sebum in LPS-treated sebocytes

In RT-PCR, the expression of LL-37 was increased in LPS-treated sebocytes and ORS cells 24 hours following the treatment with RGO (Fig. 6A). The production of sebum increased in LPS-treated sebocytes 24 hours follow-

ing the treatment with RGO ($p < 0.05$) (Fig. 6B), but not in cultured ORS cells.

DISCUSSION

RGO has been proven to have antibacterial effects and anti-inflammatory activities. Reyes et al.⁶ described that RGO interfered with the adherence of *Brucella abortus*, which

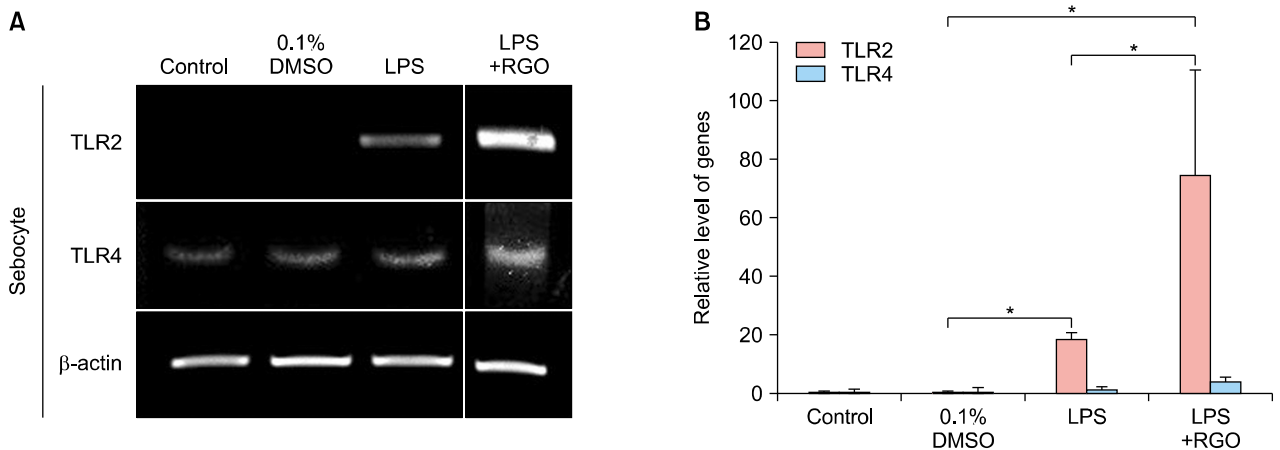


Fig. 5. (A, B) Gene expression of TLR2 was increased in LPS-treated sebocytes 24 hours following treatment with RGO. Values are presented as mean \pm standard deviation from three independent experiments ($*p < 0.05$). DMSO: dimethyl sulfoxide, LPS: lipopolysaccharide, RGO: red ginseng oil.

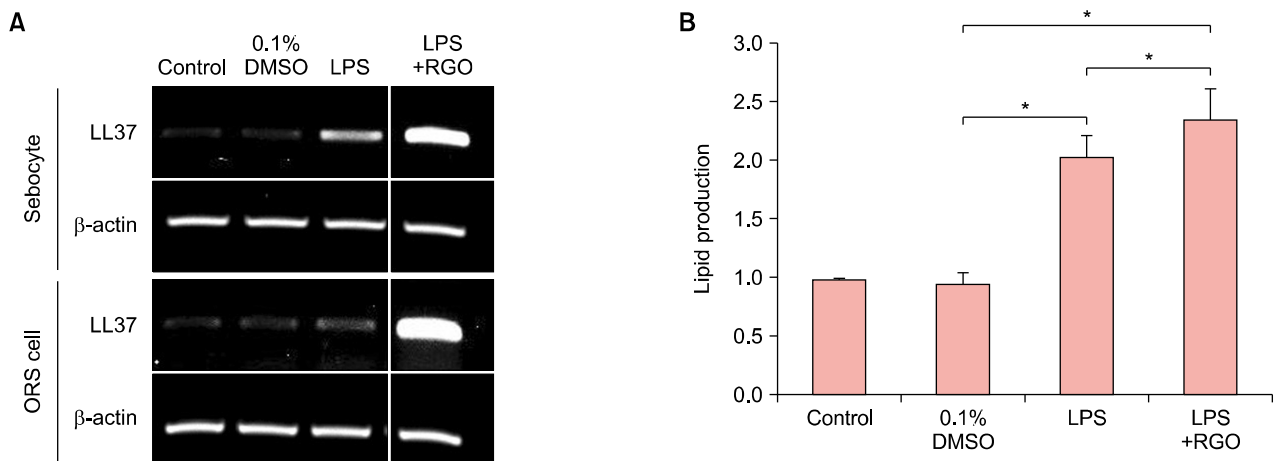


Fig. 6. (A) RGO increased the expression of LL-37 in the LPS-treated sebocytes and ORS cells. (B) RGO increased the production of sebum in LPS-treated sebocytes. Values are presented as mean \pm standard deviation from three independent experiments ($*p < 0.05$). ORS: outer root sheath, DMSO: dimethyl sulfoxide, LPS: lipopolysaccharide, RGO: red ginseng oil.

showed its potential as an anti-adhesin agent and successfully reduced bacterial uptake into macrophages. Moreover, F-actin polymerization, which is needed for the uptake of *Brucella* in macrophages, was impeded by RGO upon *B. abortus* infection. Like a study by Bak et al.⁷, the expression of the MAPKs (ERK1/2, JNK and p38 α), which also have important roles in the uptake of bacteria and remodeling of the actin cytoskeleton, was reduced in the *B. abortus*-infected macrophages. Moreover, RGO was reported to reduce p38 MAPK phosphorylation and inhibit NO production in LPS-stimulated RAW 264.7 cells⁵. Taken together, these findings show that the inhibitory effects of RGO on the MAPK-linked phagocytic signaling pathways could disrupt F-actin polymerization and the subsequent uptake of *B. abortus* in macrophages.

Reyes et al.⁶ also described that the abundant compounds identified in RGO via phytochemical analysis were linoleic acid, palmitic acid, and β -sitosterol. Palmitic acid is considered to be a new therapeutic agent for immunomodulation⁸. On the contrary, saturated fatty acid palmitic acid triggers the release of TNF- α and IL-6 from cultured primary astrocytes⁹. Linolenic acid and linoleic acid, as well as oleic acid, have been studied to have an important role in the immunomodulatory process¹⁰. Several reports indicated that β -sitosterol can be considered as an immunomodulator in pigs and utilized as an immunomodulatory agent in humans^{11,12}. Linoleic acid, β -sitosterol, and stigmasterol also diminished A β ₂₅₋₃₅ toxicity by regulating oxidative stress, apoptotic responses, and pro-inflammatory mediators¹³. Linoleic acid and stigmasterol

strongly regulated intrinsic apoptosis markers. Nonetheless, β -sitosterol blocked only the intrinsic apoptotic pathway. In our study, RGO increased apoptosis in the LPS-treated sebocytes and ORS cells. However, RGO did not increase apoptosis in the LPS-not-treated sebocytes and ORS cells. Linoleic acid, β -sitosterol, and stigmasterol downregulated iNOS and phospho-nuclear factor (NF)- κ B, but only linoleic acid and stigmasterol inhibited the expression of cyclooxygenase-2 and phospho-I κ B. In assays to assess MAPK expression for the validation of upstream signal pathways, β -sitosterol reduced the phosphorylation of p38 and ERK, but not JNK, whereas stigmasterol clearly reduced the phosphorylation of all three MAPKs, and linoleic acid clearly reduced the phosphorylation of ERK and JNK, but not p38¹³.

It was described that red ginseng marc oil (RMO) substantially inhibited the production of oxidative stress molecules including nitric oxide and reactive oxygen species in LPS-activated RAW 264.7 cells⁵. Levels of inflammatory targets such as prostaglandin E2, TNF- α , IL-1 β , and IL-6 were also reduced following the treatment with RMO. Blockade of nuclear translocation of the p65 subunit of NF- κ B was also revealed following the treatment of RMO. Gas chromatographic analysis showed that RMO contained about 10% phytosterols including sitosterol, stigmasterol, and campesterol, which may contribute to the anti-inflammatory properties. Taken together, these led to the anti-inflammatory effect of RMO in LPS-induced RAW 264.7 macrophages via the inhibition of NF- κ B transcriptional activity, possibly through the blocking of the p38 MAPK pathway. In our study, RGO increased the expression of inflammatory cytokines, including IL-1 β , IL-6, IL-8, and TNF- α , in both sebocytes and ORS cells following the treatment with LPS. However, RGO did not show an increase in the protein expression levels of IL-1 β , IL-6, IL-8, and TNF- α in the LPS-not-treated sebocytes and ORS cells. Also, RGO activated the pathway of AP-1: expression of p-c-jun and p-JNK was increased following the treatment of the LPS-pretreated sebocytes and ORS cells with RGO.

Many cathelicidins, including LL-37, are strongly upregulated during infection due to TLR activation by microbe-associated molecular patterns, such as LPS, lipoteichoic acids, and flagellin¹⁴. In addition, it was described that TLR2, which is upregulated in rosacea, promotes C/EBP α -mediated expression of CAMP and KLK5, increasing the availability of LL-37¹⁵. In our study, RGO enhanced the gene expression of TLR2 in LPS-treated sebocytes and the expression of LL-37 in the LPS-treated sebocytes and ORS cells. Moreover, LPS-pretreated sebocytes increased the production of sebum lipid following the treatment with

RGO.

In conclusion, RGO demonstrates an increase in the expression of inflammatory biomarkers in cultured sebocytes and ORS cells following the treatment with LPS. Thus, RGO might be among the aggravating factors of inflammatory acne and should be recommended to be stopped in patients with inflammatory acne.

ACKNOWLEDGMENT

The authors would like to thank Prof. Rhee MH for providing RGO.

SUPPLEMENTARY MATERIALS

Supplementary data can be found via <http://anndermatol.org/src/sm/ad-33-245-s001.pdf>.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

FUNDING SOURCE

None.

DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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