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RESEARCH ARTICLE

Effects of Gossypetin from *Hibiscus sabdariffa* on Interleukin-6 Production in *Porphyromonas gingivalis* Lipopolysaccharide-Stimulated Human Gingival Fibroblasts

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Background: Periodontal disease is a major cause of tooth loss in adults and is a representative oral disease commonly suffered by most people around the world. Mainly the proliferation of Gram-negative bacteria and secreted virulence factors cause an inflammatory response and destroy periodontal tissue. Gossypetin, isolated from *Hibiscus sabdariffa* L, is known to have various pharmacological effects, including antibacterial and anticancer activities. We aimed to confirm the anti-inflammatory effect of gossypetin through interleukin-6 (IL-6) regulation in human gingival fibroblasts (HGFs) stimulated with lipopolysaccharide (LPS) of *Porphyromonas gingivalis*, a major cause of adult periodontitis.

Methods: CCK-8 assay was performed to confirm the concentration-dependent cytotoxicity of gossypetin against HGFs. The secretion level and mRNA expression of IL-6, an inflammation-related cytokine, and the effect of gossypetin on these in HGFs stimulated with *P. gingivalis* LPS were confirmed by ELISA and qRT-PCR analysis, respectively.

Results: Up to a concentration of 100 μM gossypetin with or without *P. gingivalis* LPS, the survival rate for HGFs was maintained at over 95% and showed no toxicity. ELISA and qRT-PCR analysis results showed that *P. gingivalis* LPS increased IL-6 secretion and mRNA levels in HGFs compared to the control group. However, this increase in IL-6 was significantly down-regulated by gossypetin treatment in a dose-dependent manner. In particular, 80 μM gossypetin inhibited IL-6 production to the level of the control group.

Conclusion: These results indicated that gossypetin attenuated IL-6 production in HGFs stimulated by *P. gingivalis* LPS, which may ultimately suppress the inflammatory response in periodontal tissue. Therefore, gossypetin may have potential as a natural ingredient for the prevention and treatment of periodontal disease.

Key Words: Gingival fibroblasts, Gossypetin, Interleukin-6, Lipopolysaccharides, *Porphyromonas gingivalis*

Introduction

1. Background

Periodontal disease, including gingivitis and periodontitis, is a representative oral disease that affects many people worldwide[1)]. If the inflammatory condition in which the gingiva swells and bleeds persists, it can progress to periodontitis, which destroys the alveolar bone that supports the teeth, ultimately leading to tooth loss and systemic inflammation[1,2)]. Periodontal disease is associated with not only local risk factors such as dental plaque, a biofilm caused by oral microorganisms, but also various systemic factors such as smoking, diabetes, stress, and genetic factors[3,4)]. Therefore, compared to other oral diseases, periodontal disease is not easy to cure and is one of the diseases with a very high tendency to relapse.

Porphyromonas gingivalis, a Gram-negative anaerobic bacterium, is one of the main causative bacteria of chronic

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periodontitis, and produces many toxic factors, worsening inflammation and directly or indirectly destroying periodontal tissue[5]. Lipopolysaccharide (LPS) from *P. gingivalis* plays an important virulence factor that causes periodontal disease progression by causing an inflammatory response in gingival tissue[6]. Human gingival fibroblasts (HGFs) are the most abundant cells in gingival connective tissue and contribute to the development of periodontitis caused by periodontal pathogens such as LPS[7]. HGFs induce an inflammatory response by producing inflammatory cytokines such as tumor necrosis factor-alpha, interleukin (IL)-1β, and IL-6 through stimulation of *P. gingivalis* LPS, and it leads to the activation of osteoclasts and destroys periodontal tissue[7,8]. Previous studies have reported that HGFs increased IL-6 production in periodontitis lesions and that large amounts of IL-6 were produced locally in periodontitis patients[9,10]. Therefore, the amounts of cytokines are important in controlling inflammation in periodontal tissue.

Gossypetin, a flavonoid from *Hibiscus sabdariffa* that has been used as traditional herbal medicine due to its various pharmacological properties, is known to exhibit antioxidant, anticancer, and antibacterial effects[11-14]. However, the effects of gossypetin on periodontitis have not yet been reported.

2. Objectives

Periodontal disease, which causes an extensive inflammatory response in periodontal tissue, is the main cause of tooth loss in adults[2]. It is not easy to treat because it progresses chronically due to an imbalance between the multispecies microflora and the host immune response[15]. Therefore, it is very important to control inflammation and maintain oral care consistently during the gingivitis stage, before periodontitis progresses. To this end, it is necessary to develop products for the prevention and treatment of periodontitis using natural substances with low toxicity and high biosafety. There is a lot of evaluation of the effectiveness of various natural products related to various systemic diseases such as diabetes and cancer, but because research related to oral diseases is insufficient, the development of medicines for these diseases is not active. Accordingly, we investigated the IL-6 regulatory effect of gossypetin, a flavonoid component of *Hibiscus sabdariffa*, in HGFs stimulated with *P. gingivalis* LPS, and aimed to provide basic data for the development of future periodontitis-related products.

Materials and Methods

1. Reagents

Gossypetin (purity: ≥93%) was purchased from INDOFINE chemical Company (Hillsborough, NJ, US) and dissolved in dimethyl sulfoxide (DMSO). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline, antibiotic-antimycotic mixture containing 100 U/ml of penicillin and 100 μg/ml of streptomycin, and 0.25% trypsin-ethylenediaminetetraacetic acid solution were obtained from Gibco BRL (Grand Island, NY, USA). LPS from *P. gingivalis* was purchased from InvivoGen (San Diego, CA, USA).

2. Cell culture

HGFs were obtained from the Department of Oral Biology, College of Dentistry, Yonsei University (Seoul, Korea). HGFs were cultured in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic mixture at 37°C in a humidified atmosphere with 5% CO_2. In this experiment, HGFs from passages 3 to 6 was used.

3. Cell viability assay

HGFs (1×10^4 cells/well) were seeded into the 96-well plate and cultured in a complete medium overnight. Cells were incubated in serum-free media treated various concentrations of gossypetin with or without 1 μg/ml *P. gingivalis* LPS for 24 hours. Viability of the cells was assessed using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

4. Enzyme-linked immunosorbent assay (ELISA) assay

HGFs (2×10^5 cells/well) were plated into a 24-well plate and incubated with 1 μg/ml *P. gingivalis* LPS and various concentrations of gossypetin for 24 hours. IL-6 in the cell culture supernatants (100 μl/well) was quantified

using an ELISA kit (R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's protocols.

5. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

HGFs were plated in 60 mm culture dishes and incubated with 1 μg/ml *P. gingivalis* LPS and various concentrations of gossypetin for 24 hours. Cells were lysed and total RNA contents were isolated using a TRIzol reagent (Thermo Fisher Scientific), and total RNA was reverse transcribed into cDNA using the PrimeScrip 1st strand cDNA Synthesis Kit (Takara Bio Inc., Tokyo, Japan) according to the manufacturer's protocol. qPCR was performed with Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific) using a StepOnePlus™ Real-Time PCR system (Thermo Fisher Scientific). The sequences of primer used are as follows: IL-6 (5'-CGC CTT CGG TCC AGT TGC C-3' forward, 5'-GCC AGT GCC TCT TTG CTG CTT T-3' reverse); β-actin (5'-GAC TTA GTT GCG TTA CAC CCT TTC TTG-3' forward, 5'-CTG TCA CCT TCA CCG TTC CAG TTT T-3' reverse).

6. Statistical analysis

Data are expressed as the means±standard error from three independent experiments and analyzed by the Student's t-test and one-way analysis of variance using SPSS statistical software version 25.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was determined at p-value <0.05.

Results

1. Effect of gossypetin on the cell viability of HGFs

A CCK-8 assay was used to determine the cytotoxicity of gossypetin on HGFs. As shown in Fig. 1, cell viability was maintained above approximately 95% by treatment with 20 to 100 μM gossypetin for 24 hours compared to the control group treated with 0 μM gossypetin group. 1 μg/ml *P. gingivalis* LPS also showed no change in the viability of HGFs, and simultaneous treatment with LPS and gossypetin did not affect the cell viability (Fig. 1).

2. Effects of gossypetin on IL-6 production in *P. gingivalis* LPS-stimulated HGFs

To investigate the effects of gossypetin on the production of proinflammatory cytokine induced by *P. gingivalis* LPS in HGFs, the secretion level and mRNA expression of IL-6 were determined using ELISA and quantitative real-time PCR, respectively. As shown in Fig. 2A, the level of IL-6 in the culture medium of HGFs stimulated with *P. gingivalis* LPS increased compared to the control, and gossypetin dose-dependently inhibited the production of IL-6 induced by *P. gingivalis* LPS. In particular, 80 μM gossypetin suppressed IL-6 secretion to a level similar to that of the control group. In HGFs with *P. gingivalis* LPS treatment, the expression of IL-6 mRNA was up-regulated 4.0-fold compared to the control group, but only 1.5-, 1.3-, and 1.2-fold in the 20, 40, and 80 μM gossypetin-treated groups (Fig. 2B). Thus, gossypetin down-regulated the *P. gingivalis* LPS-increased IL-6 mRNA level in a concentration-dependent manner.

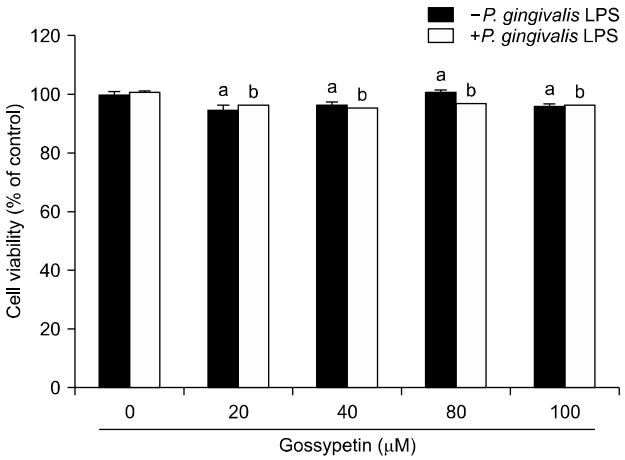

Fig. 1. Effect of gossypetin from *Hibiscus sabdariffa* on the viability of human gingival fibroblasts (HGFs). HGFs were treated with gossypetin (0, 20, 40, 80 and 100 μM) with or without 1 μg/ml *Porphyromonas gingivalis* LPS for 24 hours, and cell viability was determined by Cell Counting Kit-8 assay. Data are expressed as the mean±standard error from three independent experiments. LPS: lipopolysaccharide. [a]$p<0.05$ versus 0 μM gossypetin group (control), [b]$p<0.05$ vs 0 μM gossypetin+*P. gingivalis* LPS group.

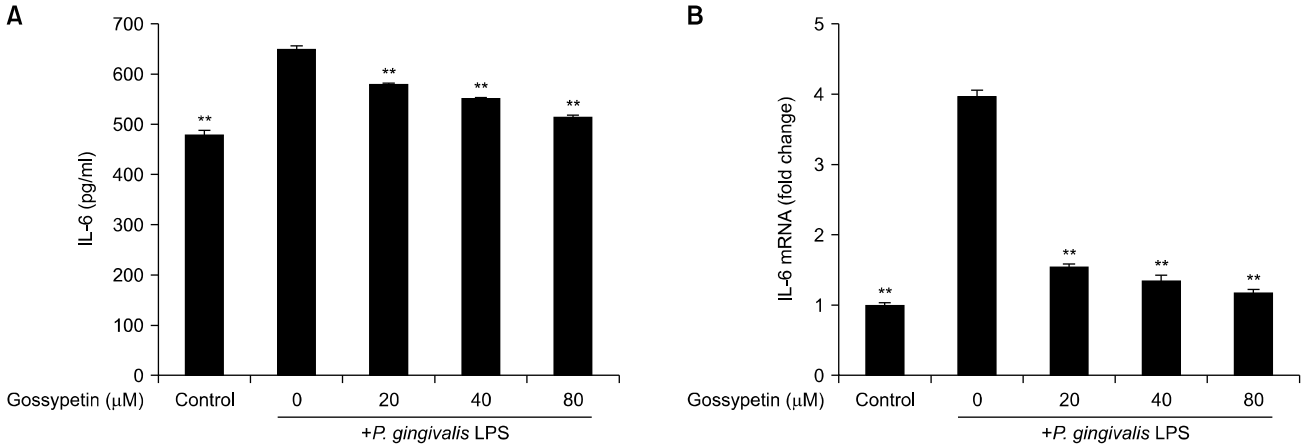

Fig. 2. Effect of gossypetin from *Hibiscus sabdariffa* on IL-6 production in *Porphyromonas gingivalis* lipopolysaccharide (LPS)-treated human gingival fibroblasts (HGFs). Cells were treated with 1 μg/ml *P. gingivalis* LPS and gossypetin (0, 20, 40, 80 μM) for 24 hours. (A) The level of IL-6 in the culture supernatant was detected using ELISA. (B) IL-6 mRNA expression was determined by qRT-PCR analysis, and bars was indicated as a fold change compared with the control. Data are expressed as the mean±standard error from three independent experiments. IL-6: interleukin-6. **$p < 0.005$ vs *P. gingivalis* LPS alone group.

Discussion

1. Interpretation and comparison to previous studies

Periodontal disease is a chronic inflammatory and infectious disease caused by the complex toxicity of various oral microorganisms[1]. *P. gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Tanerella forsythia*, which are known to cause periodontitis, are mostly anaerobic Gram-negative bacteria, and periodontal disease-related various microorganisms, including these bacteria, interact and affect each other's growth and activity[3,16]. In particular, LPS in the outer membrane of Gram-negative bacteria acts as an important endotoxin factor that stimulates the host's immune system and causes inflammation[17]. It was reported that in *P. gingivalis*, the main bacterium in adult periodontal disease, the lipid A structure of LPS activates the Toll-like receptor 4 (TLR4)-mediated nuclear factor-kappa B (NF-κB) pathway in HGFs and regulates the expression of inflammatory cytokines IL-6[18]. In addition, a previous study reported that *P. gingivalis* LPS induces an immune-inflammatory response by activating TLR2/4-mediated NF-κB/STAT3 pathways in microglial cells, and this result suggested the possibility that these periodontitis pathogens invade the central nervous system and induce neuroinflammation, causing cognitive disorders such as Alzheimer's disease[19]. In this way, bacteria and their virulence factors increased in inflammatory lesions of periodontal tissue can affect not only diseases such as cardiovascular disease and cancer, but also the central nervous system, causing neurological disorders[20]. Therefore, controlling the inflammatory response of periodontal disease can be a factor in lowering the risk of other systemic diseases.

Calprotectin, a molecule associated with inflammatory diseases, is also produced by neutrophils in inflamed periodontal tissues and induces the production of IL-6 from HGFs and the soluble form of the IL-6 receptor (sIL-6R) from macrophages[10]. The IL-6/sIL-6R complex induces the production of various periodontal tissue destructive substances from HGFs, thereby promoting periodontitis[10,21]. Therefore, because IL-6 is an important inflammatory cytokine as a biomarker in the pathophysiology of periodontitis, it is important to suppress the production of IL-6 in controlling inflammation.

2. Suggestion and limitations

In our study, IL-6 protein secretion and mRNA expression were obviously increased in HGFs by stimulation of *P. gingivalis* LPS, and were suppressed by gossypetin treatment in a concentration-dependent manner. Based on the report that *P. gingivalis* LPS activates the TLR4-mediated NF-κB pathway in HGFs and our previous study that gossypetin shows anticancer effects by downregulating the expression of phospho-NF-κB in oral cancer, it

can be expected that the efficacy of gossypetin in modulating IL-6 would be the result of a mechanism that inhibits activation of the NF-κB pathway[12,18]. Additionally, we confirmed the anti-inflammatory effect of gossypetin in an animal study using a mouse ear edema model, which is commonly used to test anti-inflammatory activity (data not shown). As a result, topical application of gossypetin significantly attenuated ear edema induced by 12-O-tetradecanoylphorbol-13-acetate, a known tumor promoter that induces the production of various inflammatory mediators through activation of mitogen-activated protein kinases and NF-κB[22]. Although the NF-κB pathway is important in the inflammatory response, our study is limited in that it did not confirm the molecular biological mechanism of the NF-κB signaling pathway of gossypetin in HGFs, so further research on this is required. Furthermore, it is necessary to confirm the action of gossypetin on the clinical symptoms and its mechanism studies through *in vivo* research using a periodontitis animal model.

3. Conclusion

We confirmed the effects of gossypetin derived from *Hibiscus sabdariffa* on the inflammatory response in periodontal disease. In HGFs stimulated with *P. gingivalis* LPS, the secretion amount and mRNA expression of IL-6, an inflammation-related cytokine, increased. However, gossypetin statistically significantly inhibited the production of IL-6 increased by *P. gingivalis* LPS in a concentration-dependent manner. Consequently, the IL-6 regulating effect of gossypetin can be expected to have the potential to be developed as a natural ingredient in periodontitis-related prevention and treatment products.

Notes

Conflict of interest

No potential conflict of interest relevant to this article was reported.

Ethical approval

Not applicable.

Author contributions

Conceptualization, funding, supervision, and writing-review & editing: Ki-Rim Kim. Experiments, Data acquisition, formal analysis, and writing–original draft: Ke Huang.

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Data availability

Data supporting the results of this study are available from the corresponding author upon reasonable request.

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