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Platycarya strobilacea leaf extract inhibits tumor necrosis factor-α production and bone loss induced by *Porphyromonas gingivalis*-derived lipopolysaccharide

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ABSTRACT

Objective: Remodeling of alveolar bone is controlled by osteoclast-mediated bone resorption and osteoblast-induced bone formation. LPS of *Porphyromonas gingivalis*, a major causative agent of periodontitis, produces proinflammatory cytokines in host immune cells, which thereby triggers osteoclastogenesis and leads to alveolar bone resorption. We investigated the anti-periodontitis potential of *Platycarya strobilacea* leaf extract (PLE), which is used as a traditional medicine in Asian countries.

Design: TNF-α levels in cell culture media were measured using a commercially available enzyme-linked immunosorbent assay kit. Osteoclast differentiation was observed by tartrate-resistant acid phosphatase staining, and the expression levels of osteoclastogenic genes were measured by quantitative real-time PCR. Bone-resorbing activity was confirmed by the resorption pit formation, gelatin zymographic, and the cathepsin K activity assays. Osteogenic differentiation was confirmed with an ALP activity assay and alizarin red S staining.

Results: PLE treatment inhibited the production of TNF-α in *P. gingivalis* LPS-stimulated RAW264.7 macrophages. In bone marrow-derived macrophages serving as osteoclast precursors, PLE treatment blocked RANKL-induced osteoclastogenesis and gene expression levels of the osteoclastogenic transcription factor NFATc1, DC-STAMP for osteoclast fusion, and cathepsin K for osteoclast activity. In addition, PLE treatment reduced the formation of resorption pits and the secretion of MMP 9 and cathepsin K from the differentiated osteoclasts. Furthermore, PLE treatment induced osteogenesis by increasing ALP activity and calcium content in pre-osteoblastic cells.

Conclusion: PLE inhibits *P. gingivalis* LPS-induced TNF-α production and bone resorption and induces bone formation. PLE may be a beneficial agent to promote oral health by inhibiting periodontitis-induced alveolar bone loss.

1. Introduction

Periodontitis is one of the most prevalent oral diseases in adults, and severe chronic periodontitis affects approximately 11.2% of the populations, particularly older age groups (Hugoson, Sjodin, & Norderyd, 2008; Kassebaum et al., 2014). Infectious periodontal pathogens activate inflammatory responses and amplify the host's immune responses in gingival tissue, causing alveolar bone resorption and subsequent tooth loss. Moreover, periodontitis and tooth loss has been reported to be closely associated with systemic diseases, including Alzheimer's disease, cardiovascular disease, and diabetes (Cerajewska, Davies, & West, 2015; Iacopino & Cutler, 2000; Lalla & Papapanou,

Abbreviations: LPS, lipopolysaccharide; TNF-α, tumor necrosis factor (TNF)-α; RANKL, receptor activator of nuclear factor-κB ligand; ALP, alkaline phosphatase; M-CSF, macrophage-colony stimulating factor; NFATc1, nuclear factor of activated T cell c1; DC-STAMP, dendritic cell-specific transmembrane protein; MMP, matrix metalloproteinase

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2011). Therefore, oral health is a very important factor in promoting quality of life. The prevention and inhibition of periodontitis can contribute to both oral and systemic health.

Although periodontitis is caused by polymicrobial synergy and dysbiosis, Porphyromonas gingivalis, a gram-negative anaerobic and asaccharolytic bacterium, is a major causative agent of periodontitis due to these bacteria's virulence and strong association with diseased sites. *P. gingivalis*-derived LPS induces the host immune response by producing proinflammatory cytokines, such as TNF-α and interleukin-1β, in gingival resident cells, including epithelial cells, fibroblasts, and macrophages. These cytokines are also produced by recruited immune cells exposed to LPS (Mysak et al., 2014). The high production of proinflammatory cytokines stimulates osteoclastogenesis directly or by inducing RANKL expression in osteoblasts and periodontal ligament fibroblasts, causing alveolar bone resorption, degradation of extracellular matrix components in periodontal tissues, and finally tooth loss (Cochran, 2008; Hofbauer et al., 1999; Wada, Maeda, Yoshimine, & Akamine, 2004).

Natural products with minimal toxicity and potent anti-inflammatory activity have been discovered to be novel biocompatible substances for the prevention and inhibition of periodontitis and alveolar bone loss (Freires, Santaella, de Cassia Orlandi Sardi, & Rosalen, 2018). *Platycarya strobilacea* Sieb. et Zucc (Juglandaceae), a small deciduous broadleaf tree, belongs to the wild walnut family. Its fruits and leaves have been used as a traditional medicine in Korea, China, and Japan, and they contain large amounts of ellagitannins, such as ellagic acid and gallic acid (Tanaka, Kirihara, Nonaka, & Nishioka, 1993; Zhang, Wang, & Xu, 2014). Recent studies reported that the fruit extract of *P. strobilacea* has antioxidant, antimicrobial, and antiaging activities (Kim et al., 2010; Zhang, Wang, & Xu, 2017); its stem bark extract, which contains a high level of polyphenols, blocked TNF-α-induced reactive oxygen species and inflammatory responses in colon epithelial cells (Babu et al., 2008).

In this study, we determined the anti-periodontal potential of *P. strobilacea* leaf extract (PLE) by investigating its inhibitory effect on the production of a proinflammatory cytokine TNF-α in macrophages stimulated with *P. gingivalis*-derived LPS. We also determined its effects on osteoclast formation and activity in RANKL-stimulated mouse bone marrow macrophages (BMMs), as well as osteoblast activation.

2. Methods

2.1. Materials

An ethanol extract of *P. strobilacea* leaves was provided by National Institute of Horticultural and Herbal Science (http://www.nihhs.go.kr:8090/extract/extract_list.asp, Wanju-gun, Jeollabuk-do, Korea). The extract was dissolved in dimethyl sulfoxide (DMSO). Minimum essential medium-alpha (α-MEM), Dulbecco's modified eagle medium (DMEM), DMEM/F12, fetal bovine serum (FBS), 0.05% Trypsin-EDTA, G418, and phosphate-buffered saline (PBS) were purchased from Gibco BRL (Grand Island, NY, USA). Histopaque-1083, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMSO, β-glycerophosphate, ascorbic acid, cetylpyridinium chloride, and alizarin red S were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant mouse soluble RANKL and M-CSF were purchased from R&D systems (Minneapolis, MN, USA). LPS from P. gingivalis was purchased from InvivoGen (San Diego, CA, USA). Collagenase A and dispase II were purchased from Roche (Basel, Switzerland). All reagents used in this study were of analytical grade.

2.2. Animals

Four-week-old male ICR and 1-day-old ICR mice were purchased from Koatech (Pyeongtaek, Korea). The mice were provided free access to a standard chow diet (Orient, Seongnam, Korea) and tap water *ad libitum*. The mice were housed under specific pathogen-free conditions with a 12-h light/dark cycle and a relative humidity of 50 ± 5% at 22 ± 2 °C. All animal studies were conducted in accordance with the experimental protocols approved by Institutional Animal Care and Use Committee of the Yonsei University College of Dentistry (IACUC Approval No. 2017-0068). All methods were carried out in accordance with relevant guidelines and regulations.

2.3. Cell culture

RAW264.7 murine macrophages were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM supplemented with 10% FBS. Mouse BMMs were isolated from the tibia of 4-week-old ICR male mice using histopaque density gradient centrifugation and cultured in α-MEM with 10% FBS and M-CSF (30 ng/mL) at 37 °C in a 5% CO_2 humidified atmosphere (Davis, 2013; Kim et al., 2015). Mouse preosteoblastic cells were obtained from the calvaria of neonatal ICR mice (age 1 day) after aseptic dissection. Mouse newborn calvarial bones were treated with 0.1% collagenase A and 0.1% dispase II enzyme solution and incubated for 30 min in a 37 °C by shaking in a water bath (Jonason & O'Keefe, 2014). The dissociated cells were washed and cultured in α-MEM medium with 10% FBS.

2.4. Cell viability

RAW264.7 cells (1×10^4 cells/well) were seeded in a 96-well plate and cultured in DMEM containing 10% FBS for 24 h. The cells were further incubated in serum-free DMEM supplemented with *P. gingivalis*-derived LPS (1 μg/mL) and indicated concentrations of PLE for 24 h. BMMs (5×10^4 cells/well) were incubated in α-MEM containing 10% FBS, M-CSF (30 ng/mL), and various concentrations of PLE for 5 days. Mouse preosteoblastic cells (4×10^3 cells/well) were incubated in α-MEM medium with 10% FBS and PLE at the indicated concentration for 7 days. Culture medium was changed every second day. The viability of RAW264.7 and preosteoblastic cells was measured by an MTT assay and that of BMMs was measured by counting the number of cells stained with hematoxylin.

2.5. TNF-α production

RAW264.7 cells were seeded in 96-well plate at the density of 1×10^4 cells/well and cultured in 10% FBS-DMEM for 24 h. The cells were incubated in serum-free DMEM with LPS (1 μg/mL) and PLE at indicated concentrations for 24 h. The levels of TNF-α in the collected culture media were measured using a Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) according to the manufacturer's instructions. Absorbance at 450 nm was measured on a microplate reader (BIO-RAD, Hercules, CA, USA).

2.6. Osteoclast differentiation

BMMs were seeded in a 96-well plate at a density of 5×10^4 cells/well and were cultured in α-MEM containing 10% FBS, M-CSF (30 ng/mL), RANKL (50 ng/mL), and the indicated concentrations of PLE for 5 days. Culture medium was changed every two days. The cells were fixed with 4% formalin and then stained for tartrate-resistant acid phosphatase (TRAP) with the Acid Phosphatase Leukocyte kit (Sigma-Aldrich) according to the manufacturer's instructions. TRAP-positive multinucleated cells with more than three nuclei were counted as the differentiated osteoclasts under light microscopy (magnification, × 100).

2.7. Osteoclastogenic gene expression

BMMs (1×10^6 cells) were incubated with M-CSF (30 ng/mL), RANKL (50 ng/mL), and PLE at indicated concentrations in a 60-mm dish for 48 h. Total RNA was isolated from cells by using the RNeasy