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Anti-inflammatory Effect of Methanol Extract from Safflower Seeds

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Periodontitis is an inflammatory disease, which destroys the connective tissue and the alveolar bone. Recently, it has been suggested that the effect of natural substances could be induced into an anti-inflammatory environment. However, the effect of Safflower seed extract (SAF-M) associated with periodontitis has not been investigated yet. Therefore, the purpose of this study was to assess the anti-inflammatory effects of SAF-M. Cytotoxicity was assessed through MTS analysis using hGF and hPDL cells. Periodontitis was induced by injecting LPS into gingival tissue on the maxillary molars of rats (45 μg LPS/one time, 3 times a week for 3 weeks). SAF-M was administered daily at 30 mg/kg and 100 mg/kg. Alveolar bone resorption was evaluated through the micro-CT. hGF and hPDL cells showed differential cytotoxicity in response to SAF-M at 5 mg/ml and 1 mg/ml concentrations. Micro-CT showed reduction of the alveolar bone resorption in the SAF-M treatment group. These results suggested that SAF-M is a potential therapeutic agent for periodontitis.

Key words: Periodontitis, Safflower seeds, Methanol extract, LPS, Alveolar bone

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Introduction

Periodontitis is inflammatory disease of the tissue around the teeth, that is one of the most common diseases in humans. Periodontitis begins with inflammation of the gingiva and further progresses to generate the absorption and destruction of the alveolar bone. Loss of alveolar bone results in a loss of teeth and swinging undermine the support of the teeth.

Immunoreactive material and inflammatory material has a plurality of outflow in the organization in the development of immune response and inflammatory pathogenesis of bacteria and toxins, destruction of periodontal tissue is chronically progressive [1]. According to the report, gram-negative bacteria play an important role in periodontitis [2]. Lipopolysaccharide (LPS) is a gram-negative bacterial outer membrane that induces the synthesis of cytokines such as Interlukin-1(IL-1), IL-6, Tumor necrosis factor (TNF) and prostaglandins from phagocytes, fibroblasts and endothelial cells [3,4]. LPS acts as a pathogen to enhance the activity of the osteoclasts, causing the loss of alveolar bone. The secretion of inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8 during the inflammatory response of periodontal tissue induces the damage to the bone by stimulating osteoclast [5]. Therefore, it is possible to alleviate and prevent inflammatory bone damage by modulation of these cytokines. Bone will maintain a dynamic state by the interaction of osteoblasts and osteoclasts. However, absorption of bone and activation is promoted function of osteoclasts to resorb bone by inflammatory stimuli [6].

Differentiation of osteoclasts responsible for bone resorption is induced by the cytokines known as RANKL [7]. Therefore, RANKL is known to be an important factor in the generation of osteoclasts [8]. Effective regulation of inflammation, can be used not only to inhibit bone destruction, but also to achieve regeneration of bone tissue through the activity of osteoblasts. When applying treatment to periodontal disease, a chronic inflammatory disease, strengthening of tissue regeneration associated with the cellular activity of periodontal tissue growth and suppression of bacteria in periodontal disease, and suppression of the production of the inflammatory response is the focus. In the treatment of gingivitis, mainly Non-steroidal anti-inflammatory drugs (NSAIDs) is used. NSAIDs has placed a basic block in the production of prostaglandin E2 (PGE2) is an inflammation parameters substances the cells produce mainly [9]. However, the production of PGE2 is stimulated by IL-1 was revealed [10]. Therefore, it is inevitable effects of periodontal tissue by cytokines produced in the previous step PGE2. For these reasons, research to develop the therapeutic effect of periodontitis using natural products recently have been actively carried out [11-16].

Based on previous studies Safflower seed was selected as the substance. Safflower is a member of the *Asteraceae* or Compositae products. The major component of the seed, includes the carbohydrate 40～50 %, fat 32～40 %, protein 11～17 %. The trace elements contains calcium and phosphorus. Safflower seed oil contains linoleic acid 60 % more essential fatty acids and oleic acid contains more than 30 %. Safflower seed has been widely used as a folk remedy in Korea to enhance bone formation and to prevent osteoporosis. In addition, safflower seed has been reported for its potential for the periodontal disease treatment, bone regeneration, osteoblast enhancement [17-21]. According to the previous studies, SAF-M not only stimulates the formation of calcified nodules but also increases the mRNA expression of alkaline phosphatase and bone sialoprotein in periodontal ligament cells and MC3T3-E1 cells. Despite the encouraging results, the effect of SAF-M in suppressing periodontal tissue damage caused by periodontitis has not yet been demonstrated. SAF-M is proven to be helpful in bone regeneration, cardiovascular system and periodontal cell regeneration [22]. However, in terms of the periodontitis treatment, it is not clear as to how it could be a help. Therefore, the purpose of this study is to assess the effect of SAF-M in periodontitis induced by LPS in the rat models.

Materials and Methods

Preparation of Agents

Safflower seeds were cultivated in Chilgokmyon, Gyeongsangbuk-do, Korea. Safflower seeds (300 g) were dried and powdered, then extracted with n-hexane (DAEJUNG, Gyeonggi-do, Korea). Using chloroform (DAEJUNG, Gyeonggi-do, Korea) and methanol (DAEJUNG, Gyeonggi-do, Korea), safflower seeds were extracted in sequence (Fig. 1). The extract was concentrated using a rotary vacuum evaporator. Methanol extract was using the experimental material. Methanol extract came out the total 15.2 g. The yield of SAF-M was 5.06 %.

Human Gingival Fibroblasts (hGF) and Human Periodontal Ligament (hPDL) Cells

The hGF cells and hPDL cells were obtained from patients for undergoing crown length procedure (CLP) treatment in the Department of Orthodontics, Wonkwang University Dental Hospital, after obtaining written informed consent from each subject, and the protocols were approved by the Wonkwang University Dental Hospital. Fragments of gingival and PDL tissue were cut in to small pieces and cultured in tissue culture dishes containing Dulbecco's

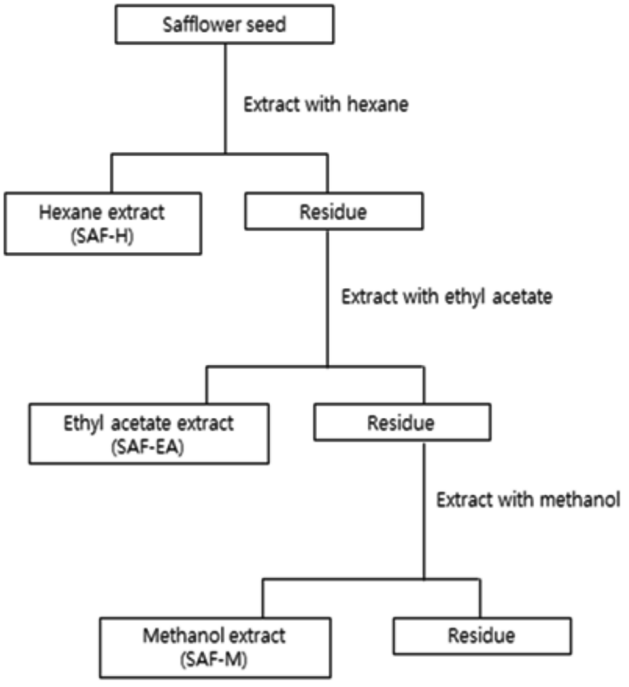

Fig. 1. Extraction process of of Safflower seeds.

modified Eagle's medium (DMEM, HyClone, USA) supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Hyclone, USA) and 1 % penicillin/streptomycin (P/S, Invitrogen, USA). Cells were incubated in 5 % carbon dioxide with the humidified atmosphere at 37 ℃. Culture medium was changed into the fresh medium every 3 days. When these cells reached confluence, the culture medium was removed and rinsed with 1 x PBS. In addition, the cell layer was treated with trypsin. The cells were subcultured by dispensing in new tissue culture plate for continued growth. In this study, cells from 6 to 8 passages were used.

MTS Assay

hGF and hPDL cells in 96-well tissue culture plate were seeded $4x10^4$ cell/well for 24 hours. Cells were treated in various concentrations (0.5, 1, 1.25, 2.5, 5 mg/ml) of SAF-M in serum-free DMEM. Treated cells were cultured for 24 hours. Then, the cytotoxicity was evaluated by MTS analysis. 490 nm wavelength was measured by enzyme-linked immuno sorbent assay (ELISA) plate reader. Cell proliferation ratio was compared to the control.

Animals and Experimental Design

Twelve Sprague-Dawley (SD) male rats were separated as the experimental group and bred to be able to ad libitum food and water. Once a day, to determine the dose of SAF-M, rats were weighed on. The experimental protocol, including the use of animals in the research, was approved by the Institutional Animal Care and Use Committee, Wonkwang University, Korea. Six groups of rats chosen randomly have healthy oral state without inflammation and normal weight gain; 1) vehicle control (inject with PBS), 2) vehicle-periodontitis (PD) (injected with LPS), 3) SAF-M 30 mg/kg control (injected with PBS) 4) SAF-M 30 mg/kg PD (injected with LPS) 5) SAF-M 100 mg/kg control (injected with PBS) and 6) SAF-M 100 mg/kg PD (injected with LPS). LPS (Sigma, St Louis, MO, USA) for periodontitis induction was injected in palatal gingiva of the right and left maxillary molar of the rats using a Hamilton syringe (Hamilton, NV, USA) as 15 uL dissolved in 1 x PBS of 990 uL the *E. coil* LPS 3 mg except for the control group. This procedure was repeated three times a week (Monday, Wednesday, and Friday) for three weeks. By injecting the same amount of 1 x PBS 15 ul, periodontitis was not induced in the negative control. By using a sonde to the SAF-M group administered diluted in DMSO 1 % (Sigma, St Louis, MO, USA), SAF-M was administered orally once a day.

Microcomputed Tomography Analysis

The alveolar bone and teeth were analyzed three dimensionally by using the Micro-CT (SkyScan 1172 Desktop MicroCT, Skyscan, Aartselaar, Belgium). The 3D digital image for each specimen was generated. 167 uA and 60 kV were used as current and voltage to obtain micro-CT images. The image of the specimen was obtained by the rotation of the 360° to 0.7° each. After scanning, reconstituted sectional slice images were acquired using a 3D analysis software. The distance from the Cemento-enamel junction (CEJ) to Alveolar bone crest (ABC) was measured at mesial root of a 2^{nd} and 3^{rd} molars and distal roots of 1^{st} and 2^{nd} molars. The location of the ABC was determined where the site of the first alveolar bone and roots are in contact with each other. The average of the four measurements were used for statistical analysis.

Statistical Analyses

Using a one-way ANOVA, the distance from CEJ to ABC was measured among the various groups. P-values less than 0.05 were determined to be statistically significant.

Results

Effect of the SAF-M on hGF and hPDL Cells

To evaluate the effect of SAF-M in hPDL and hGF cells, MTS assay was performed. Various concentrations of SAF-M were used to determine whether the cytotoxic effect of SAF-M on hGF and hPDL cells was occurred. As shown Fig. 2, the proliferation of hGF cell was gradually increased in dose-dependent manner. Also, in hPDL cell, the treatment with SAF-M resulted in the increased proliferation of dose dependency. The cytotoxicity in hPDL cell was revealed at the concentration of 5 mg/ml (Fig. 3).

Micro-CT Analyses

In vivo experiments of SAF-M, changes of the alveolar

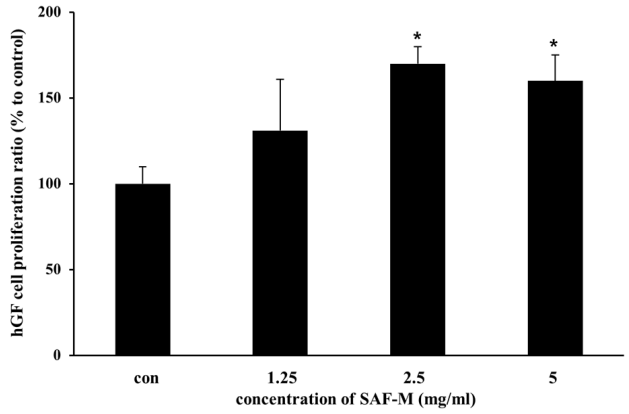

Fig. 2. Viability of hGF cells after treatment with SAF-M. Indicates that Safflower seeds methanol extract has an excellent ability to result in cell differentiation than control at a concentration of 1.25, 2.5 and 5mg/ml. In particular, at a concentration of 2.5mg/ml, the differentiation potential of the cells was two-folds higher compared to the control group.* Statistically significant difference from control group ($p<0.05$).

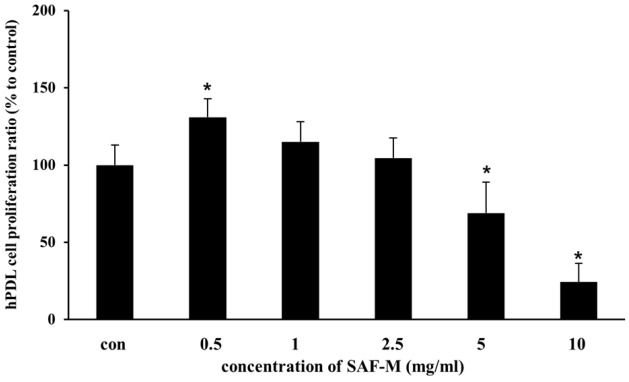

Fig. 3. Viability of hPDL cells after treatment with SAF-M. Indicates that Safflower seed methanol extract has an excellent ability to result in cell differentiation than control at a concentration of 0.5, 1 and 2.5mg/ml. In particular, at a concentration of 0.5mg/ml. * Statistically significant difference from control group ($p<0.05$).

bone were confirmed by using the micro-CT. The loss of alveolar bone was shown through a micro-CT image in each group (Fig. 3). The height of the ABC was different in each group. Height of the ABC was the highest in the control group. It can be confirmed that the height of the ABC decreased compared to the control group in the LPS group. SAF-M reduced alveolar bone loss due to LPS. It was indicated that the distance from CEJ to ABC was different in each group (Fig. 4). Distance from CEJ to ABC showed a big difference in LPS group and the control. In addition, SAF-M administered group and the LPS group showed huge

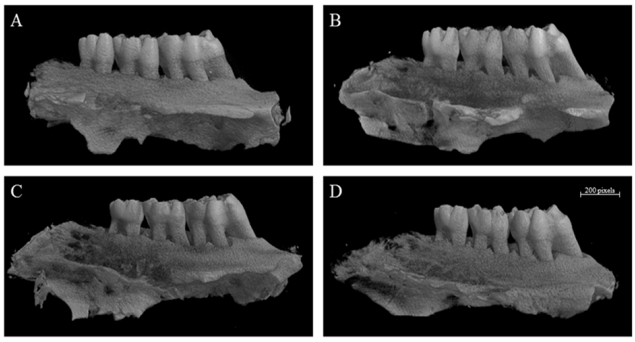

Fig. 4. The effect of the SAF-M in the loss of alveolar bone by LPS induced Micro-CT. A) group without LPS injection. B) group with LPS injection. C) LPS injected with 30mg/kg Safflower seed methanol extract. D) LPS injected with 100mg/kg Safflower seed methanol extract.

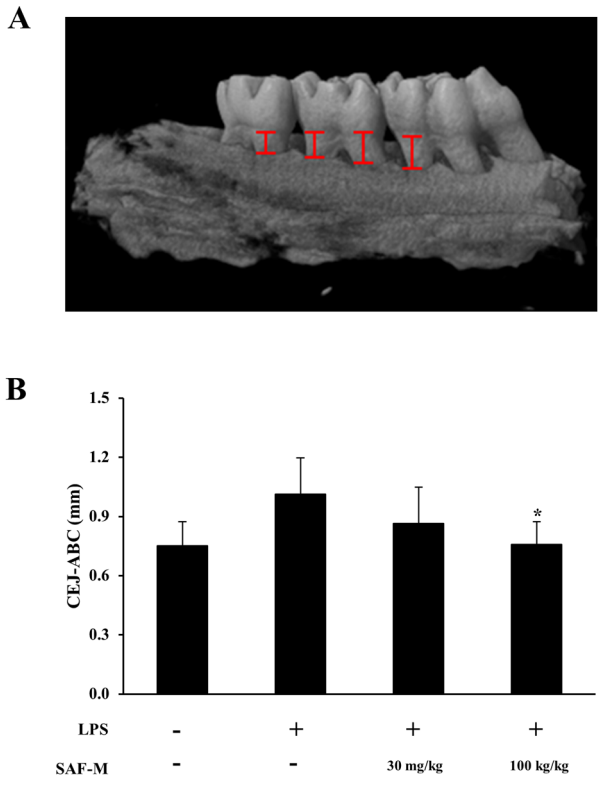

Fig. 5. The distance between ABC and CEJ. The measurement decreased reliably from LPS injected group with increasing concentration of Safflower seed methanol extract. The distance from the ABC to the CEJ was measured by a distal root of the first and second molars, as well as mesial root of the second and third molars(A). Safflower seed methanol extract administrated group a reduced loss of alveolar bone in a dose-dependent manner(B). * LPS+100mg/ml showed significant differences from group LPS ($p<0.05$).

difference in the CEJ to ABC distance. SAF-M, reduced loss of alveolar bone significantly in a dose-dependent manner.

Discussion

Periodontitis is known as a chronic inflammatory disease, which cause the alveolar bone resorption in pathological conditions. Safflower seeds, which have been traditionally used in Korea as the folk medicines related with bone tissue, has been known for its excellent effect related with the prevention of osteoporosis. However, the report about the role of SAF-M on the alveolar bone loss in periodontitis has not been studied. Therefore, this study was performed to evaluate the effect of the SAF-M on the alveolar bone loss in periodontitis induced by LPS.

The previous study was reported that the treatment of safflower seeds extracted with several organic solvent was able to dose- and time-dependently stimulate cell proliferation and differentiation of osteoblast [21]. Another research group reported that the oral treatment of methanolic extract of safflower seeds (MESS) in developing SD rats might increase the regulation of osteoblast markers, such as osteocalcin (OC) content, bone-specific alkaline phosphatase (B-ALP) activity and insulin-like growth factor 1 (IGF-1) in serum level and the length of femur and tibia on the growth parameter. When the content of MESS was analysed in the prior research, MESS contained high mineral content, such as potassium (k), calcium (Ca) and phosphorous (P). Therefore, the effect of MESS related with bone metabolism may naturally appear to be the bone formation [17].

Cytotoxicity of SAF-M appeared in hGF cell in concentration of 2.5 mg/ml, in hPDL was revealed at a concentration of 1.25 mg/ml. On the other hand, at a concentration of 2.5 mg/ml of hGF, cell activity doubled compare to the control group. This shows that the SAF-M has no toxicity to hPDL and hGF cells at low concentrations.

The micro-CT imaging were used to evaluate the alveolar bone level. Micro-CT imaging provided the form of alveolar bone. micro-CT is powerful tool to observe the morphology of the alveolar bone in the histological form accurately, to analyze the treatment of periodontitis [23].

Micro-CT analyzed that the distance between CEJ and ABC obviously increased with LPS injected group than in the control group; alveolar bone loss was induced by LPS. To be involved in the initiation of periodontitis is known that LPS [2]. Because the effect of the action appears as soon as compared to LPS of other, E coil LPS is often used in animal experimental models in particular [15]. Is regarded as the loss of alveolar bone induced by LPS, it is seen that the experimental design is well established.

Loss of alveolar bone was reduced in SAF-M infusion group compared to LPS administration group. The Ca is contained in large amounts in the SAF-M have been reported [17]. Ca is known to be essential in the re-formation of skeletal mineralization, growth, and maintenance. Inhibitory effect of SAF-M in bone resorption, can contribute to the reduction of alveolar bone loss induced by LPS.

It shows that SAF-M prevents the destruction of the periodontal ligament and gingival connective tissue. It was not known to be effective in anti-inflammatory activity SAF-M, but was found in this experiment. In conclusion, these results demonstrated that SAF-M suppressed the loss of alveolar bone induced by LPS. The regulatory mechanism of SAF-M related with lots of cytokines has not been revealed yet. Therefore, it is necessary to investigate the molecular mechanism of SAF-M in LPS-induced periodontitis.

Conflict of interest

The author declares that they have no conflicting interest.

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