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Effect of Hexane Extract of *Galla Rhois* on Inflammatory Alveolar Bone Loss

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One of functions of *Galla Rhois* (GR) is reportedly an anti-inflammatory effect on the several inflammatory diseases. However, an effect of GR related to periodontitis has not been investigated. In the present study, we examined the effect of the hexane extract of *Galla Rhois* (GR-H) on periodontitis. Cytotoxicity was assessed by MTS analysis using human gingival fibroblast (hGF) cells. Experimental periodontitis was induced by injecting *E.coli* LPS into the palatal gingiva maxillary molar thrice weekly for 3 weeks (LPS group). GR-H diluted in 1xPBS was orally administrated using a syringe at 30 mg/kg body weight and 100 mg/kg body weight once a day (GR-H group). GR-H effect on the alveolar bone loss (ABL) was digitized with a micro-CT. GR-H treatment at concentrations exceeding 0.5 mg/ml showed cytotoxic effect in hGF cells. The micro-CT among groups were presented for the different distances from cemento-enamel junction (CEJ) to alveolar bone crest (ABC). The results indicated an inhibitory effect on alveolar bone loss for orally administered GR-H in a model of LPS-induced periodontitis.

Key words: periodontitis, *Galla Rhois*, hexane extract, LPS, alveolar bone

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Introduction

There are lots of difficulties in the treatment of various diseases despite improvements of contemporary medicine and medical technology. In a diversity of therapeutic methods, the drug treatment contained a lot of artificial substances is known to produce side effects [1,2]. Therefore, the long-term treatment related with the usage of drug in the chronic diseases has been restrictedly taken.

It has been used various natural products to cure patients for a long time ago. Among lots of those, one is herb which is known as a little risk in many natural substances. In these days, many kinds of herbs have been popularly used to prevent and treat several diseases[3]. Another one is *Galla Rhois* which has been informed in traditional medicine[4]. *Galla rhois* is the gall caused by the Chinese sumac aphid, *Schlechtendalia chinensis* (Bell) from the nutgall sumac tree, *Rhus javanica* L.(Anacardiaceae). The previous studies suggested that the natural substances from *Galla Rhois* might involve with an anti-diarrhea[5], antioxidant[6], antibacterial [7] and anti-cariogenic activity[8].

Major oral diseases are dental caries and periodontal disease. Periodontal disease is significantly classified as gingivitis and periodontitis[9]. Especially, periodontitis is an inflammatory disease that is characterized by periodontal tissue destruction and alveolar bone loss. The main cause of these diseases has been reported that it is associated with gram-negative bacteria.

Most representative pathogens are Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans[10]. Gram negative bacteria has lipopolysaccharide (LPS) layer in their outer membrane of cell wall[11]. LPS can initiate immune responses by stimulating cells that reside in the periodontal tissues[12]. Consequently, production of cytokines and osteoclastogenesis were induced. In the periodontal diseases, most of studies involved with the inflammation were focused on using pharmacological agents suppressing pathways of inflammatory mediator. Pharmacological agents such as non-steroidal anti-inflammatory agents (NSAIDS) and aspirin inhibit production of prostaglandins by the cyclooxygenase kinase target.

The general initiation of immune responses is the combination of LPS with lipopoly-saccharide binding protein (LBP). And then, the CD14 is bound to form the complex of LPS/LBP/CD14. When Toll-like receptor 4 (TLR4) recognizes the complex, the signalling is started by deriving the dimerization of TLR4 by MD2. In conclusion, as mitogen-activated protein kinase (MAPK) such as p38, JNK, and ERK is activated by these signaling, cells can transmit signals and produce cytokine[12, 13]. The cytokine forms the network that can regulate inflammation by stimulating other cells[9]. Particularly, one of the functions of cytokine is inducing bone losses by promoting differentiation of osteoclasts. In this views, cytokine network plays an important role in initiation and progression of periodontal diseases[12]. As a result, therapeutic agents of periodontal disease have to suppress the secretion of pro-inflammatory cytokines or increase the anti-inflammatory cytokines. Therefore, the secretion of cytokines was the main focus in many papers associated with periodontal diseases. Some other reports proved the anti-inflammatory effects of *Galla Rhois* through experiments about immune mechanism [14,15]. However, effect of *Galla Rhois* on periodontitis have not been reported. Therefore, this study aimed to investigate the effect of *Galla Rhois* extract (GR-H) in periodontitis.

Materials and Methods

Preparation of *Galla Rhois* extract

The dried *Galla Rhois* powder (250g) was macerated in 80% methanol (MeOH) at room temperature for 5 days and filtered by using filter paper. The residue was re-extracted by the same method. In order to get the concentrates of *Galla rhois* from 80% methanol (MeOH), the whole extracts were combined and incrassated through vacuum evaporator. The concentrates was suspended in distilled water and then partitioned with *n*-hexane. After obtaining *n*-hexane fraction, the remaining aqueous layer was partitioned sequentially into ethyl acetate, and butanol (Fig. 1). All fractions were concentrated in the vaccum and we got *n*-hexane fraction (3.8g), ethyl acetate fraction (98.7g), and buthanol fraction (3.7g) respectively in that condition (Table. 1). This study used *n*-hexane fraction of *Galla Rhois* (GR-H).

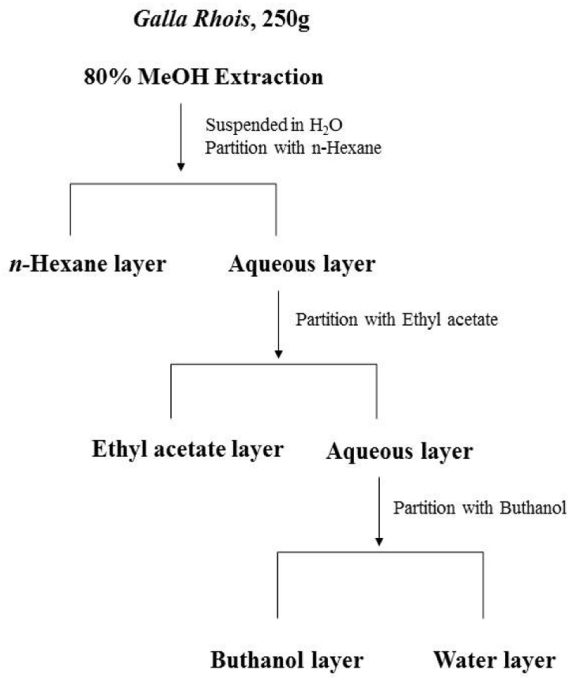

Fig. 1. Extraction process of *Galla Rhois*.

Table 1. Total organic solvent fractions of Galla Rhois

Samples	Extraction Yield(g/100g)
80%MeOH ext.	59.5
n-Hexane layer	1.5
EtOAc layer	39,4
n-BuOH layer	3.7

Cell culture of human gingival fibroblasts (hGF)

This study used human gingival fibroblast through primary culture. The hGF cells were obtained from patients who visited Wonkwang University Dental Hospital for undergoing crown length procedure (CLP) treatment. Fragments of gingival tissue were cut into small pieces and cultured in tissue culture dishes containing Dulbecco's modified Eagle's

medium (DMEM, HyClone) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone) and 1% penicillin/streptomycin (P/S, GIBCO). 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 1xPBS. 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 passage are used. Each cell culture methods are described below.

Cytotoxicity Assay

hGF was used for cytotoxicity assay. Cells were seeded at 1×10^4cells per well in 96-well tissue culture plates for 1 day. Cells were treated with the various concentration of GR-H between the 1 and 500 μg/ml in DMEM for 24 hours. After incubation, the cell proliferation was measured and analysed by Cell Titer 96™ Aqueous One solution cell proliferation assay (MTS assay). The optical density was measured by an enzyme-linked immunosorbent assay (ELISA) plate reader at 490 nm wavelength. Cell proliferation was expressed as a percentage (%) compared with the control.

Animals and experimental design

The experimental protocol, including the use of animals in the research, was approved by the Institutional Animal Care and Use Committee, Wonkwang University, Korea. 5-week-old male SD rats (~200g) were purchased from the Damul Science (Daejeon, korea). Rats were housed in individual cages. Moreover, stock diet and tap water were offered a*d libitum*. Once daily, rats were weighted to confirm proper growth and nutrition. Rats were randomly divided into six groups: (ⅰ) CON (injected with 1xPBS); (ⅱ) LPS; (ⅲ) 1xPBS + GR-H 30 mg/kg; (ⅳ) LPS + GR-H 30 mg/kg; (ⅴ) 1xPBS + GR-H 100 mg/kg; (ⅵ) LPS + GR-H 100 mg/kg (Table. 2). 5 rats per each group were anesthetized with ether inhalation. *Escherichia coli* LPS (45 μg; serotype 0111:B4 [catalog no, L2630]; Sigma), diluted at 3 μg/μL with PBS, was topically injected into the palatal gingival tissue of the upper molar of rats using hamilton syringe (Hamilton Co., PO Box 10030, Reno, NV 89520). The 1xPBS was injected in the same volume. It was performed three times per one week for 3 weeks. Before beginning LPS injection to induction periodontitis, GR-H administration started. GR-H was orally administrated at 30 mg/kg body weight and 100 mg/kg body weight using a syringe once a day. After 21 days, rats of each groups were sacrificed. Maxillary of all animals were harvested and immersed directly in 4% paraformaldehyde solution at 4℃ for 24h.

Table 2. Experimental design

Group	Administrated Agents	Agent Amount (mg/kg)	LPS injection
CON	-	-	-
LPS	-	-	+
1xPBS+GR-H 30 mg/kg	GR-H	30	-
LPS+GR-H 30 mg/kg	GR-H	30	+
1xPBS+GR-H 100 mg/kg	GR-H	100	-
LPS+GR-H 100 mg/kg	GR-H	100	+

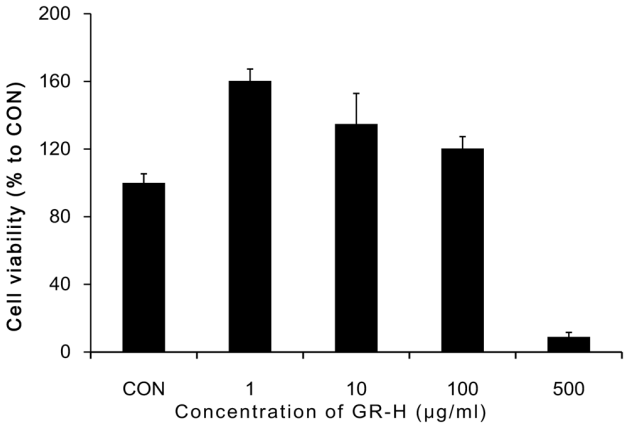

Fig. 2. Effect of GR-H on cell proliferation in hGF. hGF were incubated in the various concentration of 1, 10, 100, 500 μg/ml of GR-H for 24 hours and cell proliferation assessed by the MTS assay. *P<0.05 as compared with the non-treated group.

Microcomputed Tomography Analysis

Three-dimensional (3D) analysis of the maxillary was scanned using a Skyscan 1172 MicroCT system (SkyScan 1172; Bruker-microCT, Kontich, Belgium). 3D digitized images were produced from each specimen. Before scanning, tissues covered to protect by the para film. The maxillary were scanned at 60 kV and 167 μA with a resolution of 15.5 μm pixels. After scanning, the raw data were reconstructed by NRecon 1.6.2 (SkyScan, e2v technologies plc, Chelmsford, UK) in the from 0 to 0.1 threshold values. 3D analysis was performed through a CT-Vox (Skyscan, Aartselaar, Belgium).

Liner measurements on alveolar bone loss were taken (in mm) from cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) at the distal of first molar and second molar and the mesial at the second molar and third molar.

Statistical analysis

Cell proliferation of hGF were evaluated for significant differences using t-test. Also, statistical significance of the distance from the CEJ to the ABC was calculated by using t-test. Statistical significance was set at $P<0.05$.

Results

Cell proliferation of hGF was significantly increased at a concentration of 1 μg/ml GR-H ($P<0.05$). Although significant differences did not appear, the treatment with 10 and 100 μg/ml of GR-H increased cell proliferation. GR-H on hGF showed cytotoxic effects at concentration 0.5mg/ml or greater ($P<0.05$).

The Micro-CT image showed the levels of alveolar bone loss (ABL) in each groups (Fig. 3). CON group showed normal level of ABL by injecting 1xPBS (Fig. 3A). In LPS group, ABL is higher than it is in the CON group (Fig. 3B). Treatment of GR-H exhibited reduced level of ABL (Fig. 3C, D). Additionally, evaluation on ABL was performed by measuring distance between the CEJ and the ABC through 2D reconstruction images (Fig. 4). Mean CEJ-ABC distance

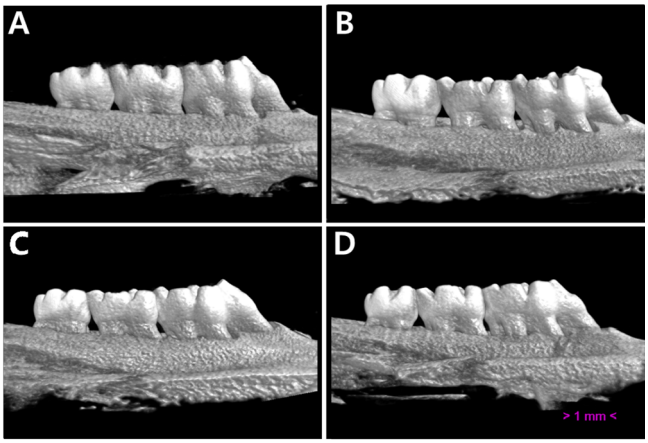

Fig. 3. Representative Micro-CT images of each group on ABL. (A) ABL of CON group with 1xPBS injection was normal level. **(B) LPS** group, LPS-induced experimental periodontitis, showed increased ABL. The oral administration group of GR-H 30 mg/kg **(C)** and 100 mg/kg **(D)** per body weight (kg) with LPS injection.

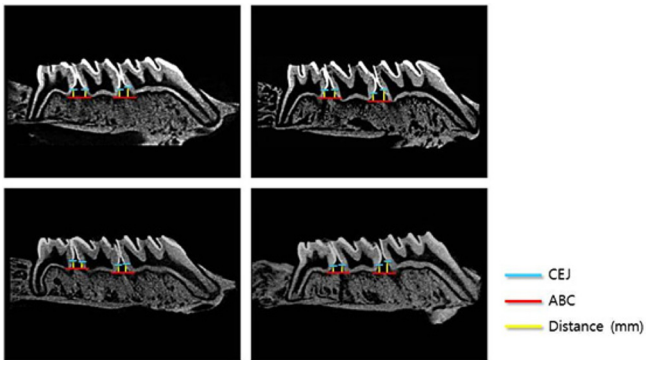

Fig. 4. Measurement of CEJ-ABC distance in 2D reconstruction images. Four lines in the measuring sites were used to assess alveolar bone loss. Linear were measured the distance from the CEJ to ABC (in mm). A: CON group, B: LPS group, C: LPS+GR-H 30mg/kg (body weight) group, C: LPS+GR-H 100 mg/kg (body weight) group.

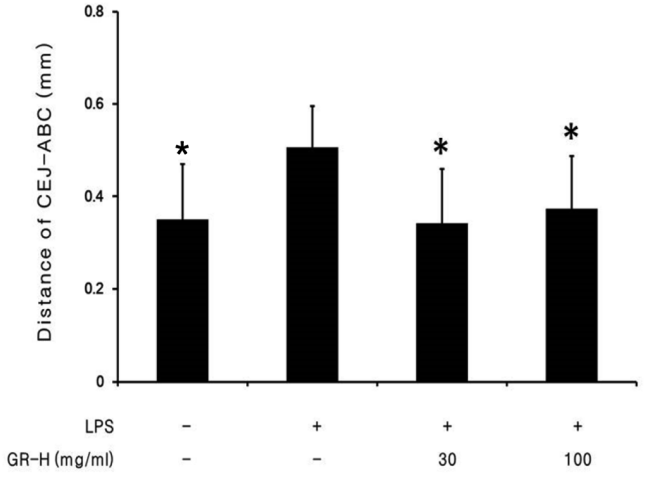

Fig. 5. Mean distance of CEJ-ABC. Administrating GR-H in the alveolar bone loss induced by LPS was significantly decreasing bone resorption than it was in the only LPS group. *$P<0.05$ as compared with the LPS group.

was 0.35 mm in CON group (Fig. 5). The mean CEJ-ABC distance was significantly increased in the LPS group than that in the CON group (0.47 mm, $P<0.05$). However, distance of ABL on LPS injection was significantly decreased to the normal level by administrating GR-H (0.36 mm and 0.39 mm respectively).

Discussion

One of the treatment related to inflammation and bone, materials having anti-inflammatory effect have been reported that bone resorption is inhibited[16]. *Galla Rhois* was proved

anti-inflammatory effect through experiment about immune mechanism[14,15]. However, effect of *Galla Rhois* has not been reported on periodontitis. Therefore, this study aimed to assess the effect of GR-H on alveolar bone loss in LPS-induced periodontitis condition.

Cytotoxic of GR-H on hGF was significantly presented at a concentration of 0.5 mg/ml ($P<0.05$). In contrast, treatment with 1 μg/ml of GR-H increased cell proliferation of hGF. This study showed that the even small amount of GR-H enhanced cell proliferation on hGF. These findings were similar to results of the other reports[17]. Cytokines are protein that initiate and regulate inflammation. Among cytokines, TNF-α and IL-6 were expressed in early periodontitis. TNF-α is correlated with the production of chemokine, IL-1β and IL-6. IL-6 is associated with osteoclastogenesis[9]. Pro-inflammatory cytokine and cell stress primarily activate JNKs and p-38[10]. Additionally, *Galla Rhois* have been reported that activation of JNK and transcription factor NF-$_{K}$B is inhibited[14]. Periodontitis, an inflammatory disease, can lead to alveolar bone destruction[12]. The bone destruction causes imbalance of between osteoblast and osteoclast by pro-inflammatory cytokines and bacterial products. Lipopolysaccharide (LPS) as bacterial products is a outer membrane component of gram-negative pathogen. It's induced production and synthesis of pro-inflammatory cytokines by stimulating host cell. However, this study used *E. coli* LPS instead of LPS derived from pathogen. Previous Researches have also used a periodontitis models by injecting *E. coli* LPS in gingiva sulcus of experimental animals[18-21]. It can induce periodontal tissue destruction as same responses of gram-negative pathogen LPS by regulating expression of target gene through any signal[10]. Therefore, effect of GR-H was evaluate from E. coli LPS-induced periodontal disease. During experimental period, body weight of rat in oral administration GR-H were compared with CON group. Body weight was almost same in each groups. Also, side effects were not showed in GR-H groups[22].

Micro-CT image is suitable for assessment of alveolar bone[23-26]. Therefore, morphology analysis on alveolar bone loss was evaluated by using micro-CT. LPS group was displayed that ABL was increased compared to the CON group. However, GR-H groups were reduced the level of ABL. Furthermore, 2D reconstruction images have provided linear data. The mean CEJ-ABC distance increased in LPS group than CON group. Alveolar bone resorption caused by injecting E.coli LPS was well established. These findings suggested that LPS induce ABL and osteoclastogenesis. In addition, the administration of GR-H decreased the ABL. Inflammatory cytokines regulate osteoclastogenesis by TNF-α and IL-6. Also, AP-1 transcription factors are essential for osteoclastogenesis. JNKs controlled AP-1[27]. These results are considered that inhibit activation of JNK decreased osteoclastogenesis by GR-H.

In summary, this study results demonstrated that alveolar bone loss was reduced by orally administrating GR-H in a model of LPS-induced periodontitis. Therefore, GR-H can be used as a therapeutic agent for periodontitis. Further studies are need to the exact mechanisms including RANK, RANKL, OPG and histological evaluation on inhibitory effects of GR-H in LPS-induced osteoclastogenesis.

Acknowledgements

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