ABSTRACT

Purpose: Aloe-emodin (AE), a natural anthraquinone abundant in aloe plants and rhubarb (Rheum rhabarbarum), has long been used to treat chronic inflammatory diseases. However, AE's underlying mechanisms in periodontal inflammation have not been fully elucidated. Acidic mammalian chitinase (AMCase) is a potential biomarker involved in bone remodeling. This study aimed to evaluate AE's effect on periodontitis in rats and investigate AMCase expression.

Methods: Eighteen Sprague-Dawley rats were separated into the following groups: healthy (group 1), disease (group 2), vehicle (group 3), AE high-dose (group 4), and AE low-dose (group 5). Porphyromonas gingivalis ligatures were placed in rats (groups 2–5) for 7 days. Groups 4 and 5 were then treated with AE for an additional 14 days. Saliva was collected from all groups, and probing pocket depth was measured in succession. Periodontal pocket tissues were subjected to histomorphometric analysis after the rats were sacrificed. Bone marrow-derived macrophages and murine macrophages were stimulated with receptor activator of nuclear factor-κB ligand (RANKL) and treated with different concentrations of AE. AMCase expression was detected from the analysis of saliva, periodontal pocket tissues, and differentiated osteoclasts.

Results: Among rats with P. gingivalis-induced periodontitis, the alveolar bone resorption levels and periodontal pocket depth were significantly reduced after treatment with AE. AMCase protein expression was significantly higher in the disease group than in the healthy control (P<0.05). However, AE inhibited periodontal inflammation by downregulating AMCase expression in saliva and periodontal pocket tissue. AE significantly reduced RANKL-stimulated osteoclastogenesis by modulating AMCase (P<0.05).

Conclusions: AE decreases alveolar bone loss and periodontal inflammation, suggesting that this natural anthraquinone has potential value as a novel therapeutic agent against periodontal disease.

Keywords: Aloe; Chitinase; Emodin; Periodontitis; Porphyromonas gingivalis; Saliva
INTRODUCTION

Periodontitis is a chronic inflammatory disease triggered by bacterial plaque accumulation along the gingiva edge that results in the destruction of bone around periodontal tissue [1,2]. It is an infectious disease that can be exacerbated or mitigated by certain risk factors [1]. Specific pathogens are responsible for the development of periodontitis in susceptible individuals [3], including Porphyromonas gingivalis, a major cause of periodontal inflammation, by releasing copious amounts of virulence factors that modulate host immune responses [1]. P. gingivalis induces an osteoclast differentiation factor, receptor activator of nuclear factor-κB ligand (RANKL) [4]. RANKL then stimulates monocytes and macrophages to secrete various inflammatory cytokines, producing various proteolytic enzymes that stimulate bone resorption [5]. Accordingly, animal models infected by this major periodontal pathogen have been utilized to investigate bone remodeling processes [6].

Aloe-emodin (AE) is one of the main bioactive ingredients common to a variety of natural herbs, including aloe vera, Polygonum multiflorum Thunb., and rhubarb (Rheum rhabarbarum) [7]. AE is a bioactive anthraquinone that has been suggested as possessing pharmacological activities, including anti-cancer, anti-microbial, and anti-inflammatory properties [7,8]. To date, some studies have confirmed AE's clinical effect on periodontitis [9,10], although the underlying mechanisms by which AE affects periodontal inflammation remain unknown.

Chitinases are hydrolytic enzymes that digest chitin. Chitin, the second most abundant polysaccharide in biodiversity, is a major component of arthropods, bacteria, and fungi [11,12]. While mammals do not produce chitin, mammals nevertheless possess chitinases, including chitotriosidase and acidic mammalian chitinase (AMCase) [13,14].

AMCase plays a crucial role in the immune response to chronic inflammatory diseases such as Crohn's disease, hepatitis, and asthma [15-17]. However, the function or mechanism of AMCase in periodontitis has not been characterized, although chitinase activity in response to periodontitis has been measured in saliva [18,19]. Moreover, the existence of AMCases has not yet been identified in periodontal pocket tissue. Therefore, the purpose of this study was to investigate AE's inhibitory effects on periodontitis and AMCase expression.

MATERIALS AND METHODS

Animals

Eighteen 6-week-old (160–180 g) Sprague-Dawley rats (Orient Bio, Seoul, Korea) were single-housed with water and food (Damool Science, Daejeon, Korea) for 7 days in plastic cages at 24°C–26°C. All animal experiments were performed consistent with the procedures of the Institutional Animal Care and Use Committee of Jeonbuk National University (JBN 2018-092).

Periodontal disease rat model and timeline design

Our experimental animal periodontitis model (Figure 1) was conducted in accordance with previously described protocols [6]. All rats were divided into the following 5 groups: healthy (G1), disease (G2), vehicle (G3), AE (Santa Cruz Biotecnology, Dallas, TX, USA), high-dose (25 mg/kg) (G4), and AE low-dose (5 mg/kg) (G5).
Ligatures (TP Orthodontics, Inc., Seoul, Korea) were soaked in *P. gingivalis* culture (10^9/mL) overnight. *P. gingivalis* ligatures were positioned in each rat’s left maxillary first and second molars for 7 days and removed on day 7 (Figure 1A). Intramuscular injection for anesthesia was 20 mg/kg Zoletil 50 (Laboratoires Virbac, Carros, France). Rats in the disease control group (G2) were sacrificed on day 7; at this time, saliva, periodontal pocket tissue, and alveolar bone samples were collected, and the periodontal pocket depth was measured. The treatment groups (G4 and G5) were treated with AE daily for another 14 days, and the periodontal pocket depth was measured daily between day 14 and day 17 (Figures 1B and 2B). The rats in G1 and G3–5 were sacrificed on day 20, and saliva, periodontal pocket tissue, and alveolar bone samples were collected.

**Preparation of saliva and periodontal pocket tissue samples**

Rats were anesthetized, and their mouths were washed with water. Saliva was collected with cotton balls changed every 10 minutes after drying their mouths using a cotton-tipped applicator. These cotton balls were then cooled in an icebox, and the saliva was collected via centrifuging at 10,000 rpm at 4°C for 10 minutes. Periodontal pocket depth was measured daily from day 14 to day 17 (Figure 2B). Periodontal pocket tissue samples were collected from the left side of each rat’s first and second maxillary molars after sacrifice. The periodontal pocket tissue was washed three times with phosphate-buffered saline (PBS) and cut into small pieces. To remove red blood cells, the tissue was washed with Gey’s salt solution for 30 minutes and washed again with PBS. The tissue was transferred to a lysis buffer containing sodium deoxycholate (0.25%), ethylenediaminetetraacetic acid (EDTA; 1 mM), NaCl (150 mM), Tris-HCl (50 mM), aprotinin (5 µg/mL), NaVO₃ (1 mM), pepstatin (1 µg/mL), NaF (20 mM), NP-40 (1%) and phenylmethylsulfonyl fluoride (1 mM) for 2 hours at 0°C. The tissue
lysate was then centrifuged (13,200 rpm) at 4°C for 10 minutes. All samples were kept at −80°C before analysis.

**Histomorphometric analysis**

Maxilla samples were isolated and fixed overnight in 0.1 M phosphate buffer, pH 7.4, containing 10% formalin. Then, samples were decalcified by EDTA (10% solution) for 3 weeks. The decalcified and dehydrated specimens were embedded in paraffin. Sections of 6 µm were cut from the embedded specimens, stained with hematoxylin and eosin, and observed using light microscopy. The distance between the cementoenamel junction (CEJ) and the alveolar bone crest (ABC) was measured in the proximal area of the upper molars (Figure 2A).

**Culture of macrophages**

RAW264.7 (ATCC, Manassas, VA, USA) cells as murine macrophages were grown in Dulbecco’s Modified Eagle’s Medium supplemented with fetal bovine serum (FBS) (10%), penicillin (100 U/mL), and streptomycin (100 U/mL) at 37°C and 5% CO₂. The tibiae and
femurs from 6-week-old mice (Damool Science, Daejeon, Korea) were used to isolate bone marrow-derived macrophages (BMMs). After harvesting adherent cells, BMMs were cultured for 3 days in Minimum Essential Medium Eagle α-modification with 10% FBS, macrophage colony-stimulating factor (30 ng/mL), and RANKL (50 ng/mL).

**3-(4,5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) assay**

Macrophage cells (RAW264.7) were incubated at 5×10^3 cells/well and grown overnight in 10% FBS under various concentrations of AE. After 72 hours, the washed cells were treated by MTT (100 µg/mL) at 37°C for 2 hours. Absorbance was measured at 540 nm after the cells were suspended by dimethyl sulfoxide (200 µL).

**Western blots**

Macrophage cells (RAW264.7 and BMM) were washed with PBS. The cells were then lysed in ice-cold buffer containing sodium deoxycholate (0.25%), EDTA (1 mM), NaCl (150 mM), Tris-HCl (50 mM), aprotinin (5 µg/mL), NaVO₄ (1 mM), pepstatin (1 µg/mL), NaF (20 mM), NP-40 (1%), and phenylmethylsulfonyl fluoride (1 mM). After 30 minutes, the lysates were prepared by centrifugation (16,100 rcf at 4°C for 10 minutes). After separation of proteins via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%), the transferred polyvinylidene difluoride membrane was blocked in nonfat skim milk (5%) for 1 hour using Tris-buffered saline with Tween-20 (0.25%) at 16°C (TBST). Then, the membrane was incubated for 18 hours at 4°C with anti-AMCase (Santa Cruz Biotechnology, Dallas, TX, USA) (1:2,000 to 1:3,000 dilution) in the nonfat skim milk in TBST. The membrane was then incubated with a secondary antibody (1:2,500 to 1:3,000 dilution) in dry milk in TBST for 2 hours. Blots were developed using the enhanced chemiluminescence western blotting agent (Sigma-Aldrich, Inc., St. Louis, MO, USA).

**Statistical analysis**

All data were analyzed using the Dunnett multiple comparison test and SPSS software version 13.0 (SPSS Inc., Chicago, IL, USA). The data are presented as the mean±standard error of at least 3 replicates. Values were considered statistically significant if P<0.05.

**RESULTS**

**Reduction of periodontal bone loss and pocket depth by AE**

To investigate AE's therapeutic effects on alveolar bone loss and periodontal inflammation, an animal model with P. gingivalis-induced periodontitis was used according to the timeline illustrated in Figure 1. The maxilla was collected after each rat was sacrificed. The distance between the CEJ and ABC in the molars was then measured (Figure 2A). The histomorphometric analysis showed that alveolar bone loss was significantly inhibited in both the high-dose and low-dose AE groups compared to the disease group (P<0.05). Notably, a more pronounced effect was observed in the high-dose AE group (Figure 2A). As expected, more bone loss and periodontal inflammation were observed in the P. gingivalis-infected group than in the healthy control and AE-treated groups. To evaluate AE’s effects on periodontal inflammation, clinical pocket depth was also measured between day 14 and day 17 (Figure 2B). Compared with the disease and vehicle groups, considerably shallower pockets were observed in both the high-dose and low-dose AE groups.
Suppression of AMCase expression by AE in saliva and periodontal pocket tissue

To investigate the role of the AMCase in saliva, whole saliva (around 300 µL) was collected from rats after adjunctive AE treatment. AMCase protein levels were examined using western blots. After *P. gingivalis* ligature insertion, AMCase expression was enhanced in the disease group compared to the healthy group. However, both high-dose AE and low-dose AE significantly decreased AMCase expression in the rat saliva (Figure 3A). AE’s effects on levels of AMCase in periodontal pocket tissue were also explored. AMCase protein expression was enhanced in the disease group compared to the healthy control group. AMCase expression was significantly lower after treatment with high-dose and low-dose AE (Figure 3B). These results suggest that AE inhibits ligature-stimulated periodontitis by down-regulating AMCase protein levels in saliva and periodontal pocket tissue.

Cytotoxicity of AE in RAW264.7 cells

To examine AE’s effects on cell viability, cells were pretreated under various conditions of AE (2.5, 5, 10, 15, or 20 µM) for 72 hours and evaluated by MTT assay. We observed that AE up to a maximum concentration of 15 µM had no cytotoxic effects. After pretreatment, cytotoxic effects began to manifest with 20 µM AE (Figure 4).

Suppression of AMCase activation by AE in differentiated osteoclasts

To identify AE’s effects on osteoclastogenesis, RANKL-stimulated macrophage cells (RAW264.7) were treated with different concentrations of AE. AMCase expression in RAW264.7 has been confirmed by western blot using anti-AMCase for specific detection. According to previous research [17], AMCase expression was strongly increased by RANKL stimulation. Interestingly, we observed that AMCase expression was significantly downregulated by 5 and 10 µM AE treatment, suggesting that AE may inhibit osteoclast differentiation by modulating AMCase (Figure 5A).
To investigate AE’s effects on early-stage RANKL-induced osteoclastogenesis in BMMs, RANKL-stimulated cells were treated with 10 µM AE between days 1 and 4. AMCase expression was significantly increased by RANKL stimulation (Figure 5B). However, treatment with 10 µM AE significantly reduced AMCase expression levels between days 2 and 4. This result highlights AE’s important role in the early stages of osteoclastogenesis.

DISCUSSION

This study found that orally administered AE inhibited bone loss in a pathogen-stimulated periodontitis animal model. The anthraquinone derivative AE has long been used to treat
various ailments related to chronic inflammation [7,8,20]. Indeed, we determined that probing pocket depth and bone resorption significantly decreased in the periodontitis model after AE was given. To the best of our knowledge, this was the first study to investigate AE’s effects on \textit{P. gingivalis}-induced periodontitis in rats. More importantly, our data suggested that AMCase, a potential bone remodeling biomarker, is associated with periodontal inflammation [21].

Bone remodeling is a physiologic balance between osteogenesis (or bone formation via osteoblasts) and osteoclastogenesis (or bone resorption via osteoclasts) [22,23]. Osteoclast differentiation is regulated by crucial cytokines such as RANKL that induce an essential signal for osteoclast formation and survival [22,23]. Accordingly, RANKL-stimulated macrophage cells, including BMMs or RAW264.7, have been widely used as in vitro models to screen for bone resorption activity [24,25]. In a previous paper, we determined that RANKL-stimulated RAW264.7 could differentiate into osteoclasts, and omega-3 together with aspirin inhibited RANKL-induced osteoclastogenesis by downregulating an osteoclast-specific gene and pro-inflammatory genes [6]. Another study reported that AE dose-dependently suppressed pro-inflammatory mediators including iNOS, COX-2, and prostaglandin E2 in RAW264.7 [20].

In accordance with previous research [21], we found that AE suppressed RANKL-induced osteoclastogenesis by inhibiting AMCase marker gene expression, suggesting that AMCase is closely associated with bone remodeling (Figure 5). In this study, AE treatment, compared to no treatment (i.e., disease or vehicle group), significantly reduced alveolar bone resorption and periodontal inflammation in a rat periodontal model (Figure 2). In sum, these results suggest that AE’s effect on RANKL-induced osteoclastogenesis is associated with a reduction in periodontal disease through downregulation of AMCase.

Saliva is a vital biological fluid that maintains oral and general health [26]. Several studies have sought to demonstrate saliva’s utility as a diagnostic biomarker for diabetes, cardiovascular diseases, and lung diseases [26-28]. Current research programs aim to develop “high-impact,” “quick,” and “easy” screening tests such as HIV viral tests and cholesterol monitoring [19,29]. Most recently, non-invasive on-site point-of-care saliva-based diagnostic tools have been explored as a means of identifying periodontal diseases through relevant biomarkers, including C-reactive protein, matrix metalloproteinase, and chitinases [19,30,31].

While studies have focused on the activity of salivary chitinases [18,32], much less work has been done on the levels of AMCase associated with periodontitis [33]. In our study, the protein expression of AMCase was directly validated from saliva and periodontal pocket tissue. Since periodontal pocket tissue contains both interactive proteins from the host and infectious pathogenic bacteria, a direct analysis of protein biomarkers taken from the periodontal pocket may prove to be a precise method of diagnosing periodontal diseases in the early stage and grade [31].

Previous studies have demonstrated that chitinase activity increases in periodontitis and decreases after treatment [18,32]. Consistent with those observations, we found that salivary AMCase protein expression was substantially enhanced in periodontal disease compared to the healthy group (Figure 3). AMCase expression in saliva and periodontal pocket tissue was significantly reduced after AE treatment, suggesting that AE reduces periodontal inflammation by downregulating AMCase. One of the limitations of our pilot study is its small sample size. Future research should confirm these findings with a larger number of samples in animals and humans.
Most recently, novel anti-chitinase drugs have been developed to treat chronic lung inflammation in antigen-induced animal models and human clinical trials [34,35]. Since potential new therapeutics have also been actively under development in the periodontal field [36], AE would represent a novel adjunct therapeutic drug targeting AMCase modulation.

In conclusion, our study investigated a new therapeutic for periodontal inflammation that modulates the host bone response through AE treatment. This preclinical study also evaluated the role of AMCase expression in periodontal inflammation. In the *P. gingivalis*-induced periodontitis rat model, AMCase expression was higher in the disease group than in the healthy control group, and AE inhibited the expression of AMCase in saliva and periodontal pocket tissue. Moreover, AE also inhibited RANKL-stimulated osteoclastogenesis by modulating AMCase, thereby preventing bone resorption. These results suggest that AE has potential value as a novel therapeutic agent against periodontal disease.

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