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Zanthoxylum piperitum reversed alveolar bone loss of periodontitis via regulation of bone remodeling-related factors

Mi Hye Kim[a], Hye Ji Lee[a], Jung-Chul Park[b], Jongki Hong[c], Woong Mo Yang[a,*]

[a] Department of Convergence Korean Medical Science, College of Korean Medicine, Kyung Hee University, Seoul, Republic of Korea
[b] Department of Periodontology, College of Dentistry, Dankook University, Cheonan, Republic of Korea
[c] College of Pharmacy, Kyung Hee University, Seoul, Republic of Korea

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ABSTRACT

Ethnopharmacological relevance: *Zanthoxylum piperitum* (ZP) has been used to prevent toothache in East Asia.
Aim of study: In this study, we investigated the effects of ZP on periodontitis along with alveolar bone loss.
Materials and methods: Twenty-eight male Sprague-Dawley rats were assigned into 4 groups; non-ligated (NOR), ligated and treated vehicle (CTR), ligated and treated 1 mg/mL ZP (ZP1), and ligated and treated 100 mg/mL ZP (ZP100). Sterilized 3-0 nylon ligature was placed into the subgingival sulcus around the both sides of mandibular first molar. After topical application of 1 and 100 mg/mL ZP for 2 weeks, mandibles was removed for histology. In addition, SaOS-2 osteoblast cells were treated 1, 10 and 100 μg/mL ZP for 24 h to analyze the expressions of alveolar bone-related markers.
Results: Several alveolar bone resorption pits, which indicate cementum demineralization were decreased by ZP treatment. Topical ZP treatment inhibited periodontitis-induced alveolar bone loss. In addition, there were significant reduction of osteoclastic activities following topical ZP treatment in periodontium. The expression of RANKL was decreased in SaOS-2 osteoblast cells by treating ZP, while that of OPG was increased. ZP treatment increased the expressions of Runx2 and Osterix in SaOS-2 cells.
Conclusion: In summary, ZP treatment inhibited alveolar bone loss as well as maintained the integrity of periodontal structures via regulation of bone remodeling. ZP may be a therapeutic target for treating periodontitis.

1. Introduction

Periodontal disease is an important factor in health systems worldwide. Poor periodontal health eventually lead to tooth loss up to 20% of the adult population (Petersen et al., 2005). This disease is characterized by bone destruction as well as inflammation of the periodontium and connective tissues disruption (Liu et al., 2013). The alveolar bone loss is a typical appearance of periodontitis (Behl et al., 2008; Graves et al., 2011). In terms of alveolar bone, bone remodeling is simultaneously controlled by osteoclasts and osteoblasts. Osteoclasts conduct bone resorption while bone formation is induced by osteoblasts (Matsuo, 2009). Periodontal disease cause disruption of dual actions of osteoclasts and osteoblasts, leading to alveolar bone loss (Kim et al., 2014).

Some of modulating drugs such as anti-proteinases, anti-inflammatory and bone-sparing agents are used for treating periodontitis (Elavarasu et al., 2012). Due to recognition that periodontitis is a bacterially host immune-inflammatory responses to pathogenic plaque, various host modulation therapy has been used to suppress the destructive component of the host response (Preshaw, 2008). The use of anti-inflammatory drugs inhibits the secretion of prostaglandins and inflammatory cytokines. However, unexpected adverse effects including gastrointestinal symptoms, bleeding and acute renal and hepatic impairment have been appeared (Bhatavadekar and Williams, 2009). In addition, antibiotics therapy is capable of reducing pathogenic bacteria; however; damaged alveolar bone loss is not completely recovered (Walker, 1996). Anti-resorptive drugs, which are extensively used to inhibit the alveolar bone loss (ABL), have been reported to

Abbreviations: ABL, alveolar bone loss; CTR, ligatured and vehicle-treated group; H & E, hematoxylin and eosin; NOR, non-ligatured and non-treated group; OPG, osteoprotegerin; Runx2, runt-related transcription factor 2; RANKL, nuclear factor kappa-B ligand; TRAP, tartrate resistant acid phosphatase; ZP, 50% ethanol extract of *Zanthoxylum piperitum*; ZP1, ligatured and ZP 1 mg/mL-treated group; ZP100, ligatured and ZP 100 mg/mL-treated group

* Corresponding author.
E-mail address: wmyang@khu.ac.kr (W.M. Yang).

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present the risk of osteonecrosis of the jaw (Ponzetti et al., 2015). Because of these undesirable side effects, persistent efforts have been made to develop the alternative agents for periodontitis.

Zanthoxylum piperitum DC (Rutaceae) is an important food condiment as well as herbal medicine (Jiang and Kubota, 2001). The root of *Z. piperitum* is reported to treat carminative, stomachic, and anthelmintic problems (Lee and Lim, 2009; Li et al., 2010). Especially, *Z. piperitum* has been traditionally used for prevention of toothache in East Asia (Epple et al., 2001; Zeng and Zhao, 2015; Zhang et al., 2015). Previous studies have reported that *Zanthoxylum* species contain various benzo[*c*]phenanthridines, coumarins, lignans, flavonoids, quinolines, benzenoids, and triterpenoids (Ahsan et al., 2000; Tantapakul et al., 2012). These compounds serve anti-platelet aggregation, anti-HIV, and anti-inflammatory activities (Chen et al., 2009; Cheng et al., 2005; Sheen et al., 1994). Among them, Quercitrin, one of the flavonoids of *Z. piperitum*, was studied to have the regenerative effects on periodontal soft tissues by inhibiting extracellular matrix and inflammation-related cytokines in human gingival fibroblasts and mesenchymal stem cells (Gomez-Florit et al., 2015). In addition, isozanthpodocarpin B, dimeric lignan derived from *Zanthoxylum* species, exhibited anti-inflammatory effects in LPS stimulated RAW 264.7 cells (Li et al., 2015). Furthermore, *Z. piperitum* and its compound, protocatechuic acid, was reported to have protective properties against oxidative stress in rats (Hur et al., 2003).

Nevertheless, there is no previous study to investigate the effect of *Z. piperitum* on periodontal disease. In this study, we demonstrated the inhibitory effect of *Z. piperitum* against periodontitis using ligature-induced experimental rat model. In addition, the expressions of bone remodeling-related markers such as runt-related transcription factor 2 (Runx2), Osterix, receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG) were determined in osteoblast-like SaOS-2 cells.

2. Materials and methods

2.1. Preparation of Zanthoxylum piperitum extract

The dried fruit of *Z. piperitum* DC was purchased from oriental medicine market (Jung-do Herb, Seoul, Korea). The herbs (100 g) were soaked in 50% ethanol (1 L) at room temperature for 24 h. The ethanol extract was filtered through filter paper and concentrated in a rotary vacuum evaporator for 30 min to remove the ethanolic base. The concentrated extracts were freeze-dried. The dried powder (called ZP) obtained from fruits of *Z. piperitum* DC was 5.61 g (5.61% yield). A voucher specimen (PerioH-ZP050) was deposited at our laboratory.

2.2. Quality evaluation of ZP

The quantification of hyperoside (3-O-galactoside of quercetin) and quercitrin in ZP was performed by high-performance liquid chromatography with a diode-array detector (HPLC-DAD, Agilent 1200 series). Hyperoside and quercitrin were used as markers of ZP (Hur et al., 2001; Cho et al., 2003). Fifty mg ZP was dissolved in 1 mL 70% methanol and sonicated for 30 min. After filtering through a 0.2 μm filter polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA), the resulting solution was 4-fold diluted with 70% methanol. Ten microliters of final solution was injected into a HPLC system equipped with a SHISEIDO CAPCELL PAK C18 (250×4.6 mm, 5 μm) column. The mobile phase consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile (80:20) with 0.5 mL/min flow rate at 25 °C. The wavelength for measurement was set at 250 nm. The procedures were analyzed in triplicate by comparing the retention times, and the amounts were quantified using the peak area of the standard curves. The correlation coefficients (R^2) for both compounds were greater than 0.997, indicating excellent linearity. The linear range of hyperoside and quercitrin was 2.5–100 and 5–250 μg/mL, respectively. The concentrations of hyperoside and quercitrin in ZP were 40.342 ± 0.13 and 23.209 ± 0.04 μg/mL, respectively. The relative standard deviations of both compounds were less than 8.07%.

2.3. Preparation of ZP gel

To facilitate topical application, carboxymethylcellulose was used for enhancing the viscosity (Khoshneviszadeh et al., 2014; Sun et al., 2013). The dried ZP powder was added to distilled water in accordance with decided concentrations (1 and 100 mg/mL). 1% carboxymethylcellulose was transferred into the ZP solution (1 g carboxymethylcellulose dissolved in 100 mL solution). The base of gel (vehicle) was also supplied by the same method without ZP component.

2.4. Experimental design

7 weeks aged Sprague-Dawley male rats (250 ± 50 g), which are commonly used for ligature-induced periodontal experiment (Fu and He, 2013), were purchased from RAONBIO Inc. (Yongin, Korea). They were housed in an air-conditioned room (20 ± 2 °C temperature and 50 ± 5% humidity) under a 12 h light/dark cycle with food and water freely available. All experiments were conducted according to the guidelines of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by Committee on Care and Use of Laboratory Animals of the Kyung Hee University (KHUASP(SE)-14-029).

The rats were randomly placed in 4 groups (n=7); (i) NOR, non-ligatured and non-treated, (ii) CTR, ligatured and vehicle-treated, (iii) ZP1, ligatured and ZP 1 mg/mL-treated and (iv) ZP100, ligatured and ZP 100 mg/mL-treated. Under general anesthesia with intraperitoneal injection of a tiletamine/zolazepam mixture (Zoletil 50; Virbac Lab, Carros cedex, France), the experimental periodontitis was induced by a ligature (sterilized 3-0 nylon) placement into the subgingival sulcus around the both sides of mandibular first molar of rats, except NOR group (Kuhr et al., 2004). Immediately after ligature placement surgery, 100 μL of vehicle and ZP gel were topically applied to palatal gingival sulcus of both maxillary first molars. The rats were sacrificed after continuous 14 days treatment once per day. Both side of mandibles were resected to be fixed in 10% neutralized formalin for 18 h.

2.5. Measurement of alveolar bone loss

The fixed right mandibles were de-fleshed with boiled water and stained with 1% aqueous methylene blue (Sigma, MO, USA) to differentiate bone from tooth. Each root surface of each molar (n=7) were calculated by a computerized densitometry system Image J (NIH, Bethesda, MD, USA). The total ABL was summed from the buccal tooth surfaces and subtracting the values of the right maxilla, in mm.

2.6. Histological and Tartrate resistant acid phosphatase (TRAP) analysis

The fixed left mandibles were dehydrated with ethanol and xylene following decalcification for 2 months with 0.1 M ethylene diamine tetraacetic acid aqueous solution. The tissues were embedded in paraffin. To observe histological changes, 7 μm cut sections were stained with hematoxylin and eosin (H & E). The integrity of periodontal structures were showed at ×40 and 200 magnification. To observe osteoclastic activity, 7 μm cut sections were stained with tartrate resistant acid phosphatase (TRAP) and naphthol ASBI phosphate (Sigma, St. Louis, MO, USA). To classify the TRAP-stained cells and nuclei, hemotoxylin staining was performed. The digital images were obtained from Leica Application Suite (LAS) microscope software (Leica Microsystems, Buffalo Grove, IL, USA). The multinuclear cells were showed at ×200.