Inhibitory effects of French pine bark extract, Pycnogenol®, on alveolar bone resorption and on the osteoclast differentiation

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ABSTRACT

Pycnogenol® (PYC) is a standardized bark extract from French maritime pine (*Pinus pinaster* Aiton). We examined the inhibitory effects of PYC on alveolar bone resorption, which is a characteristic feature of periodontitis, induced by *Porphyromonas gingivalis* (*P. gingivalis*) and osteoclast differentiation. In rat periodontitis model, rats were divided into four groups: group A served as the non-infected control, group B was infected orally with *P. gingivalis* ATCC 33277, group C was administered PYC in the diet (0.025%: w/w), and group D was infected with *P. gingivalis* and administered PYC. Administration of PYC along with *P. gingivalis* infection significantly reduced alveolar bone resorption. Treatment of *P. gingivalis* with 1 μg/ml PYC reduced the number of viable bacterial cells. Addition of PYC to epithelial cells inhibited adhesion and invasion by *P. gingivalis*. The effect of PYC on osteoclast formation was confirmed by tartrate-resistant acid phosphatase (TRAP) staining. PYC treatment significantly inhibited osteoclast formation. Addition of PYC (1-100 μg/ml) to purified osteoclasts culture induced cell apoptosis. These results suggest that PYC may prevent alveolar bone resorption through its antibacterial activity against *P. gingivalis* and by suppressing osteoclastogenesis. Therefore, PYC may be useful as a therapeutic and preventative agent for bone diseases such as periodontitis.

INTRODUCTION

Periodontal diseases are chronic inflammatory disorders of bacterial origin characterized by loss of alveolar bone and destruction of connective tissue supporting the teeth (Cochran, 2008; Highfield, 2009). These diseases are mixed infections induced by a specific group of Gram-negative anaerobic bacteria (Feng *et al*., 2006; Haffajee *et al*., 1994). Among over 700 bacterial species that have been identified in the oral cavity, *Porphyromonas gingivalis* (*P. gingivalis*), a black-pigmented Gram-negative anaerobe, has been recognized as one of the most important causative microorganism in chronic periodontitis. The initial event in most infectious diseases involves the adhesion of pathogens to host tissues and subsequent invasion by the pathogens. *P. gingivalis* can adhere to and invade epithelial cells, which is thought to facilitate retention in the oral environment and contribute to immune evasion and tissue destruction. Furthermore, *P. gingivalis* produces a number of virulence factors including fimbriae, adhesins, capsular polysaccharide and lipopolysaccharide as well as numerous proteases that contribute to host colonization, immune defense system neutralization and periodontal tissue destruction (Hamada *et al*., 2007; Holt *et al*., 2005).

Plant polyphenols have attracted a good deal of attention for their potential human health benefits and clinical uses (Pandey *et al*., 2009). Extensive studies have been

carried out and indicated that intake of polyphenols may prevent some types of cancers, cardiovascular diseases and other disorders associated with oxidative stress, owing to their antioxidant properties (Scalbert *et al*., 2005; El Gharras, 2009). Furthermore, polyphenols have been reported to possess antimicrobial and anti-inflammatory characteristics (Scalbert *et al*., 2005; El Gharras, 2009). Because periodontal diseases are associated with pathogenic bacteria and causing tissue inflammation around the teeth, polyphenols may be of interest as preventive and therapeutic agents.

Pycnogenol® (PYC) is a standardized bark extract of the French maritime pine *Pinus pinaster* (formerly known as *Pinus maritime*) Aiton, which grows in the coastal region of southwest France. Approximately 65-75% of PYC extract consists of procyanidins comprising catechin and epicatechin subunits of varying chain lengths. Other constituents include polyphenolic monomers, phenolic or cinnamic acids and their glycosides (Rohdewald, 2002). The quality of this extract is specified in the United States Pharmacopeia (USP 28). The whole extract and individual fraction have been shown to have strong antioxidant capacity in *in vitro* cultured cells, perfused organs and *in vivo* models (Packer *et al*., 1999). Cho *et al*. reported that an extract from *Pinus maritime* inhibited the gene expression of proinflammatory cytokines in RAW 264.7 cells (Cho *et al*., 2006). In human studies, PYC was also demonstrated diverse

anti-inflammatory actions. Double-blind, placebo-controlled studies in asthma patients showed reduced plasma or urine leukotriene concentrations after PYC supplementation, while asthma symptom scores and pulmonary function improved (Rohdewald, 2002; Hosseini *et al*., 2001; Lau *et al*., 2004). Another report showed that symptoms of chronic venous insufficiency such as pain and edema decreased (Arcangeli, 2000). With respect to the effectiveness of PYC for periodontal diseases, little information is available except the report by Kimbrough *et al*. about PYC-containing chewing gum, which minimized gingival bleeding and plaque formation (Kimbrough *et al*., 2002). The purpose of the present study was to investigate the antibacterial effects of PYC against *P. gingivalis*, the inhibitory effects on the experimental periodontitis in rats induced by *P. gingivalis* and *in vitro* osteoclast differentiation in order to evaluated PYC as a preventive and therapeutic agent for periodontal diseases.

MATERIALS AND METHODS

Preparation of PYC

PYC powder (French maritime pine bark extract) was provided by Tradepia Co. (Tokyo, Japan). PYC was dissolved in distilled water, sterilized through a 0.45 μm filtration

membrane and serially diluted with phosphate-buffered saline (PBS, pH 7.4) or cell culture media. In order to determine the effect of the extract on experimental periodontitis in rats, PYC was mixed into standardized diet as a hard briquette at 0.025% (wt/wt) concentration by Oriental Yeast Co., Ltd. (Tokyo, Japan), the dose level of which was estimated to equivalent to a human intake level.

Bacterial strains and growth conditions

The bacterial strain used in this study was *P. gingivalis* ATCC 33277. *P. gingivalis* was grown in brain heart infusion broth (BHI broth; Becton Dickinson Co., Sparks, MD, USA) supplemented with 5 mg/ml yeast extract, 5 μg/ml hemin and 1 μg/ml vitamin K1 (BHIY-HK broth) at 37°C for 18 h under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2).

Experimental periodontitis in rats

Twenty-four male, specific-pathogen free, 4-week-old, Sprague-Dawley rats, each weighing approximately 80 g, were obtained from a commercial farm (Nihon SLC, Shizuoka, Japan). The rats were provided with water and fed a standardized diet or standardized diet supplemented with PYC as hard briquettes, both provided by Oriental

Yeast. The animals were maintained in a 12-h light-dark cycle (lights on 08:00–20:00) at a temperature of 22°C and relative humidity of 50%. Rats were given sulfamethoxazole (1 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) and trimethoprim (200 μg/ml; Sigma) in drinking water for 4 days to reduce the original oral flora, followed by a 3-day antibiotic-free period before being challenged with *P. gingivalis*. The rats were divided randomly into the following four groups of six rats each (Fig. 1). Group A received only 5% carboxymethylcellulose (CMC; Sigma). Group B was infected orally with *P. gingivalis* ATCC 33277 suspended in 5% CMC, and each rat received 0.5 ml (3.0×10^9 colony-forming units (CFU)/ml) by oral gavage five times at 48-h intervals. Group C was administered PYC in the diet, and Group D was infected orally with *P. gingivalis* and administered PYC. Each group of six animals was kept in a cage throughout the experimental period to facilitate successful isolation. Thirty-four days after the final infection, all rats were sacrificed under general anesthesia. The experimental procedures in this study were reviewed and approved by the Committee of Ethics on Animal Experiments of Kanagawa Dental University and were carried out under the guidelines for animal experimentation of Kanagawa Dental University, which are in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Measurement of alveolar bone resorption

The upper jaws were defleshed by treatment after 10 min in an autoclave at 15 pounds/inch^2, and then immersed in 3% hydrogen peroxide, rinsed, air-dried and stained with 1% methylene blue. The distance between the cemento-enamel junction (CEJ) and the alveolar bone crest (ABC) around the maxillary molars was measured at seven buccal sites per rat and horizontal alveolar bone resorption was evaluated. Measurements were made using a dissecting microscope (×40) fitted with a digital high-definition system (digital HD microscope VH-7000; Keyence, Osaka, Japan) that was standardized to provide measurements in millimeters (Fig. 3A).

Antibacterial activity against *P. gingivalis*

Exponentially growing *P. gingivalis* cells were washed and suspended in PBS to an optical density of 1.0 at 600 nm, which equated to approximately 4×10^9 CFU/ml. Ten μl of bacterial suspension was exposed for 1, 10, and 60 min to 1 ml of 1 μg/ml, 10 μg/ml, 100 μg/ml or 1000 μg/ml PYC, or the same volume of PBS as a control. After the PYC treatment, *P. gingivalis* cells suspension was serially diluted and 100 μl of each dilution was spread onto a BHIY-HK sheep blood agar plate. The number of CFU was determined after 7 days' incubation in an anaerobic atmosphere. Each experiment was

carried out three times, and the results were shown with the mean value of the experiments. Bactericidal activity was defined as a reduction in viable bacteria of $\geq 3\log_{10}$ CFU/ml at any of the incubation periods tested.

Human gingival epithelial cell cultures

Primary cultures of human gingival epithelial cells (HGECs) were prepared as follows. Briefly, healthy gingival tissues were collected during oral surgery from dental patients for the removal of impacted third molars with their informed consent. The tissues were digested with 0.4% dispase (Godoshusei, Tokyo, Japan) overnight at 4°C. The surface epithelium was separated and dissociated further in 0.05% trypsin solution (GIBCO, Buffalo, NY, USA) into single-cell suspensions. The cells were collected and suspended in keratinocyte growth medium (KGM; LONZA, Walkersville, MD, USA) and incubated at 37°C under 5% CO_2 until confluent. The cells were suspended in trypsin and seeded at 4×10^4 cell per well in a 24-well culture with KGM. After 48-h incubation, the cells were grown to near confluency (10^5 cells/well) and used for adherence and invasion assays.

Adherence and invasion assays

Adherence to and invasion of HGECs by *P. gingivalis* was assessed using an antibiotics protection assay. The *P. gingivalis* culture was centrifuged, washed with KGM and resuspended in KGM at a final concentration of 10^9 cells/ml. Bacterial suspensions were added to confluent HGEC monolayers at a multiplicity of infection (MOI) of 100 and incubated at 37°C in 5% CO_2 for 90 min. After incubation, unattached bacteria were removed by washing of the monolayers twice with sterile PBS. HGECs were lysed in 1 ml of sterile distilled water per well and incubated for 30 min. Lysates were serially diluted, plated on BHIY-HK blood agar plates and incubated anaerobically at 37°C for 7 days.

Invasion of HGECs by *P. gingivalis* was quantified by determining the number of CFU recovered after antibiotic treatment. Confluent HGEC monolayers, infected with *P. gingivalis* (MOI = 100) and incubated for 90 min, were washed with PBS twice. External adherent bacteria were killed by incubating with KGM containing gentamicin (300 μg/ml) and metronidazole (200 μg/ml) for an additional 60 min. The monolayers were rinsed again with PBS before lysis in sterile water. Internalized bacteria were enumerated on BHIY-HK blood agar plates. The inhibitory effects of PYC on *P. gingivalis* adherence and invasion to HGECs were examined by adding various concentrations of PYC to the HGEC cultures 60 min prior to bacterial infection. All

assays were performed in triplicate.

Cell viability assay

Cell viability in MC3T3-G2/PA6 (PA6) cells (RIKEN Cell Bank, Tsukuba, Japan) was determined by the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega Co., Madison, WI, USA) according to the manufacture's instructions. In brief, PA6 cells were placed in 96-well plates at a concentration of 1×10^4 cells/well and were grown in alpha-Minimum essential medium (α-MEM) (GIBCO) containing 20% fetal bovine serum (FBS; SAFC Biosciences, Denver, CO, USA) to sub-confluence. The cells were then treated with 0.1 μg/ml, 1 μg/ml, 10 μg/ml, 100 μg/ml or 500 μg/ml PYC. After 48 h incubation, the absorbance was measured at 490 nm. Results are expressed as the mean ± standard deviation (SD) of the relative percentage of viability compared with negative controls without PYC (n = 5).

Osteoclast differentiation assay

PA6 cells were used in this assay. The function of the cells was similar to bone marrow-derived stromal cells that promote osteoclast differentiation when co-cultured with spleen cells in the presence of 1α,25-dehydroxyvitamin D3 [1α,25-$(OH)_2D_3$] and

dexamethasone (Udagawa *et al.,* 1989). Bone marrow cells (1.5×10^5 cells/well), obtained from the tibiae of 5- to 8-week-old BALB/c mice, and PA6 cells (1.5×10^4 cells/well) were co-cultured in α-MEM with 20% FBS for 7 days in the presence of 10^{-8} M 1α,25-$(OH)_2D_3$ (Calcitriol; Wako Pure Chemical Industries, Osaka, Japan) and 10^{-8} M dexamethasone (Sigma) in 48-well plates in a 5% CO_2 atmosphere. The inhibitory effects of PYC on osteoclast formation was assessed by adding PYC to the culture at the indicated final concentrations. After 7 days incubation, the co-cultured cells were stained for tartrate-resistant acid phosphatase (TRAP), a marker enzyme of osteoclasts. TRAP-positive multinucleated cells containing three of more nuclei were counted as osteoclasts. Results were expressed as the means ± SD of triplicate cultures.

Survival assay

BALB/c mouse bone marrow cells (1.5×10^7 cells) and PA6 cells (1.5×10^6 cells) were co-cultured in α-MEM supplemented with 10% FBS, 1α,25-$(OH)_2D_3$ (10^{-8} M) and prostaglandin E_2 (10^{-7} M; MP Biomedicals, LLC, Solon, OH, USA) in 100 mm dishes pre-coated with type I collagen gel (cell matrix type-IA; Nitta Gelatin, Inc., Osaka, Japan) for 7 days. All cells were removed from dishes by treatment with 0.2% collagenase (Sigma), replaced in 24 well culture plates and incubated for 6 h. After

incubation, PA6 cells were removed by treatment with 0.05% trypsin-EDTA (GIBCO) for 5 min. The purity of osteoclasts in this preparation was about 95%. Purified osteoclasts spontaneously die due to apoptosis; however, receptor activator of NF-κB ligand (RANKL) promotes the survival of purified osteoclasts. The effect of PYC on the survival of osteoclasts stimulated with RANKL was examined. Purified osteoclasts were then further cultured for 48 h with vehicle (control) and the 200 ng/ml RANKL (Sigma) in the presence or absence of PYC. After incubation, cells were fixed and stained for TRAP and TRAP-positive multinucleated cells containing more than three nuclei were counted as viable osteoclasts. Results were expressed as the mean ± SD of triplicate cultures.

Statistical analysis

Differences between two groups were analyzed using Student's *t* test. For comparison of more than two groups, analysis of variance (ANOVA) was performed followed by Tukey's test as a post hoc test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Antibacterial activity against *P. gingivalis*

The treatment of *P. gingivalis* with 1-1000 μg/ml PYC reduced the number of viable cells in a dose-dependent and time-dependent manner. After 10 μg/ml, 100 μg/ml or 1000 μg/ml PYC treatment for 10 min, the number of CFU of *P. gingivalis* decreased by 28.4%, 70.4% and 94.1%, respectively. Bactericidal activity against *P. gingivalis* was only observed with 1000 μg/ml PYC treatment for 60 min (Fig. 2), and the number of CFU of *P. gingivalis* decreased from $3.63 \pm 0.12 \times 10^7$ to $1.02 \pm 0.06 \times 10^3$. After 60-min treatment, 1 μg/ml, 10 μg/ml and 100 μg/ml PYC reduced viable cell numbers by 18.5%, 43.5% and 76.5%, respectively.

Inhibitory effects of PYC on *P. gingivalis* adherence and invasion

Adherence to and invasion of HGEC by *P. gingivalis* was 5.74 ± 0.67% at an MOI of 1:100 (Table 1). Addition of PYC to the culture reduced the number of adherent bacterial cells in a dose-dependent manner. In particular, 10 μg/ml and 100 μg/ml PYC significantly inhibited bacterial adhesion by 64.5% and 84.2%, respectively ($p < 0.01$). Moreover, a remarkable inhibitory effect of PYC on *P. gingivalis* invasion was observed. The addition of 1 μg/ml PYC to the culture resulted in a significant decrease in invasion

efficiency by 0.25 ± 0.05% (76.2% relative inhibition) compared with the efficiency without PYC ($p < 0.01$).

Alveolar bone resorption

No rats showed any obvious signs of systemic illness throughout the study period. The animals had similar body weights at the beginning of the experiment and mean body weights exhibited a similar rate of increase during the experimental period. At the end of the experimental period, mean body weights in groups A, B, C and D were 481.3 ± 14.7 g, 478.0 ± 30.3 g, 462.0 ± 25.0 g and 446.0 ± 28.4 g, respectively.

To evaluate the effect of PYC on *P. gingivalis*-induced alveolar bone resorption, bone level differences among the rat groups were determined. Figure 3B shows the mean [± standard error of the mean (SEM)] CEJ-ABC distance at each of the seven measurement sites. As alveolar bone decreased, the CEJ-ABC distance increased. The bone levels of groups A, B, C and D were 0.36 ± 0.03 mm, 0.47 ± 0.03 mm, 0.36 ± 0.02 mm, and 0.39 ± 0.04 mm, respectively. The bone loss level of group B, which was infected with *P. gingivalis*, was 30.6% greater than that in the non-infected control group ($p < 0.01$), indicating that *P. gingivalis* infection caused bone resorption. The bone level of the *P. gingivalis*-infected plus PYC administration group (group D) was

significantly less than that of the *P. gingivalis*-infected group ($p < 0.01$), and the level was similar to that of the non-infected controls. Since administration of PYC itself had no effect on alveolar bone resorption, these results indicated that administration of PYC inhibited *P. gingivalis*-induced alveolar bone resorption.

Effect of PYC on cell viability

We performed MTS assays to assess the effect of PYC on PA6 cell viability and to determine the appropriate concentration for treating cell cultures. Compared with untreated cells, no significant effect on cell viability was shown at a concentration of 100 μg/ml PYC (Fig. 4). Therefore, PYC was used at a concentration of less than 100 μg/ml in the osteoclast formation assay.

Inhibitory effect of PYC on osteoclast differentiation

Co-cultures of PA6 cells and mouse bone marrow cells produced TRAP-positive multinucleated osteoclasts in the presence of 1α,25-$(OH)_2D_3$ and dexamethasone. Treatment with PYC, at concentrations of 1 μg/ml, 10 μg/ml and 100 μg/ml, significantly inhibited osteoclast formation by 34.5%, 53.4% and 100.0%, respectively ($p < 0.01$, Fig. 5). Furthermore, addition of 100 μg/ml PYC resulted in complete

inhibition of the formation of TRAP-positive cells as well as multinucleated osteoclasts (Fig. 5A).

PYC inhibits the survival of osteoclasts

Purified osteoclasts died spontaneously after an additional 48-h incubation because of apoptosis. On the other hand, treatment of the osteoclasts with RANKL (200 ng/ml) significantly reduced the number of apoptotic cells and prolonged the cell survival of osteoclasts, while maintaining their enormous cell form (Fig. 6A, B). The number of TRAP-positive multinucleated osteoclasts with RANKL was approximately 8.4-fold higher than that of the control without RANKL treatment. The addition of PYC (1-100 μg/ml) to the cell cultures with RANKL significantly reduced the number of survived osteoclasts in a dose-dependent manner ($p < 0.01$).

DISCUSSION

Periodontal diseases are chronic inflammatory disorders that lead to the destruction of tooth-supporting tissues, including the periodontal ligaments and alveolar bone. These diseases are caused by a specific group of Gram-negative anaerobic bacteria (Cochran,

2008; Highfield, 2009; Feng *et al*., 2006; Haffajee *et al*., 1994). Among periodontopathic bacteria, *P. gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Tannerella forsythia* have been reported to cause destructive periodontitis in rodents when these microorganisms were given orally (Baker *et al*., 1994; Evans *et al*., 1992; Schreiner *et al*., 2003; Sharma *et al*., 2005). This model is useful to examine the preventive and therapeutic effects of drugs and natural compounds, such as plant extracts, on periodontal destruction induced by periodontopathic bacteria.

In this study, we investigated the inhibitory effects of PYC on alveolar bone resorption using a rat experimental periodontitis model. PYC administration significantly reduced bone resorption induced by *P. gingivalis* ($p < 0.01$, Fig. 3B). Since plant polyphenols possess potent antimicrobial properties (Pandey *et al*., 2009; Scalbert *et al*., 2005; El Gharras, 2009), the antibacterial effects of PYC against *P. gingivalis* were examined. The treatment of *P. gingivalis* with 1-1000 μg/ml PYC reduced the number of viable cells in a dose-dependent and a time-dependent manner. Torras *et al*. examined the antimicrobial activity of PYC and found that PYC possessed marked bacteriostatic activity against a broad range of microorganisms, including cariogenic and periodontopathic bacteria; however, the activity was not microbicidal (Torras *et al*., 2005). In our study, bactericidal activity against *P. gingivalis* was observed with 1000

μg/ml PYC treatment for 60 min (Fig. 2), showing that over 99.9% of bacteria were killed. The study by Sakanaka *et al*. showed that green tea polyphenols inhibited the growth of *P. gingivalis* at concentrations of 250-1000 μg/ml, using the purified six components of Japanese tea (Sakanaka *et al*., 1996). Purified constituents of PYC may have potent inhibitory effects on *P. gingivalis* growth.

Adhesion to host tissue surfaces is the first step in bacterial infection. *P. gingivalis* possesses various adhesion molecules, such as fimbriae, outer membrane proteins and polysaccharides (Lamont *et al.*, 2000). Adherent bacteria begin to grow and colonize the oral cavity to cause inflammation. As shown in Table 1, PYC (French pine bark extract) significantly inhibited adherence to and invasion of human gingival epithelial cells by *P. gingivalis*. Tea extracts were also reported to possess both antibacterial and antiadherence activities against *P. gingivalis* (Sakanaka *et al*., 1996; Zhao *et al*., 2013). In our study, a remarkable inhibitory effect of PYC was observed on *P. gingivalis* invasion. Gingival epithelial cells react to bacterial challenges by signaling host responses and integrating innate and acquired immune responses (Dale, 2002). Invasive bacteria can escape from the host immune system and may lead host cell to destruction. Therefore, the inhibitory effects of PYC on adherence to and invasion of epithelial cells by *P. gingivalis* may act as protective measures against oral infection.

Recent studies have suggested that the production of reactive oxygen species (ROS) may play an important role in the pathogenesis of periodontitis (D'Aiuto *et al*., 2010; Chapple *et al*., 2007). Stimulation of bacterial antigens induced a large amount of ROS production from polymorphonuclear leukocytes, which resulted in the promotion of oxidative damage to gingival tissue, periodontal ligaments and alveolar bone. Tomofuji *et al*. (Tomofuji *et al*., 2009a) suggested that increased levels of oxidative stress due to excessive production of ROS are involved in the pathogenesis of periodontitis, and the intake of vitamin C, an antioxidant, down-regulated ligature-induced periodontitis lesions and gene expression associated with inflammation. They also demonstrated that systemic administration of a cocoa-enriched diet, which diminished periodontitis-induced oxidative stress, inhibited alveolar bone loss and polymorphonuclear leukocytes infiltration in a ligature-induced periodontitis model (Tomofuji *et al*., 2009b). PYC is known to possess potent antioxidant properties (Rohdewald, 2002; Packer *et al*., 1999). Grimm *et al*. (Grimm *et al*., 2004) observed that PYC metabolite M1 displayed superoxide scavenging activity. Therefore, the antioxidant activity of PYC administered orally may have prevented bone resorption in the present study.

Inflammation and bone resorption are main features of periodontal diseases.

Bacterial elements stimulate a local inflammatory reaction in gingival tissues, including induction of various proinflammatory cytokines production. Interleukin (IL)-1 and tumor necrosis factor-alpha (TNF-α) play important roles to stimulate adhesion molecules expression which is a necessary step for leukocyte to leave the vascular system, the induction of enzymes that degrade connective tissue, and eventually lead to alveolar bone loss (Graves *et al*., 2003; Garlet *et al*., 2006). Accumulated evidence demonstrates anti-inflammatory activity of PYC. Pretreatment of LPS-stimulated RAW 264.7 cells with PYC reduced both the production of IL-1β ant its mRNA levels (Cho *et al*., 2006). Peng *et al.* demonstrated that inhibitory effect of PYC on the induction of adhesion molecules in TNF-α-treated human vascular endothelial cells (Peng *et al.*, 2000). The matrix degrading enzyme, such as matrix metalloproteinase 2 (MMP-2) and MMP-9, is thought to lead connective tissue breakdown. The research of Grimm *et al.* showed human plasma samples after five days intake of PYC suppressed MMP-9 release from monocytes stimulated with LPS (Grimm *et al.*, 2006). The anti-inflammatory activity of PYC may contribute to suppress alveolar bone resorption.

Bone resorption can be inhibited in several ways. By reducing the differentiation rate of osteoclasts, the number of functionally activated osteoclasts for bone resorption can be decreased. It is also possible to inhibit resorptive activity by inducing the

apoptosis of mature osteoclasts. Therefore, we examined the inhibitory effect of PYC on osteoclastogenesis using an *in vitro* cell culture assay. Addition of PYC to the culture significantly inhibited osteoclast formation at concentrations of 100 μg/ml, 10 μg/ml and 1 μg/ml (Fig. 5). Furthermore, the same concentration of PYC showed significant inhibition of the number of surviving mature osteoclasts stimulated with RANKL (Fig. 6). RANKL is a key factor in regulating the process of osteoclast differentiation and maintaining the survival of mature osteoclasts. The binding of RANKL to its receptor, RANK, triggers the activation of cytoplasmic tumor necrosis factor receptor-associated factor 6, which subsequently induces the activation of mitogen-activated protein kinases and transcription factors including NF-κB, AP-1 and NFATc1 (Boyle *et al*., 2003; Lee *et al*., 2003). These transcription factors play a critical role in the regulation of genes involved in osteoclast differentiation and the bone resorptive activity of mature osteoclasts (Takayanagi *et al*., 2002). Lin *et al*. demonstrated that (-)-epigallocatechin gallate (EGCG), a major component of the green tea polyphenols, inhibited osteoclast differentiation in osteoclast precursor RAW 264.7 cells through the activation of NF-κB (Lin *et al*., 2009). Since a significant inhibitory effect on cell viability of PA6 cells was not observed with PYC, the same suppression of signal transduction may result in a decrease in the number of osteoclast formed. EGCG was also reported to inhibit

survival of osteoclasts differentiated from RAW 264.7 cells (Yun *et al*., 2007). In this study, PYC remarkably inhibited alveolar bone resorption in rat periodontitis. Since PYC was administered orally, the effectiveness of PYC for rat periodontitis may be caused by the constituents or the metabolites derived from PYC. Further examination is necessary, however, the findings from osteoclast culture assays may suggest that the ability of PYC to regulate osteoclast formation and survival is associated to the inhibition of alveolar bone resorption by PYC.

In conclusion, we demonstrated that PYC possesses an inhibitory effect on alveolar bone resorption in rat periodontitis induced by *P. gingivalis* infection. We considered that PYC suppressed alveolar bone resorption by its antibacterial activity against *P. gingivalis* and by inhibiting osteoclast differentiation and survival. Therefore, PYC may be useful as a therapeutic and preventative agent for bone diseases such as periodontitis.

Acknowledgments

Conflict of interests

The authors have no conflicts of interest to declare.

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FIGURE LEGENDS

Figure 1. Experimental procedure. Rats were divided into four groups (n = 6/group). Group A, control (no challenge with *P. gingivalis*); group B received an oral challenge with *P. gingivalis*; group C, administered Pycnogenol® mixed in the diet; and group D, administered Pycnogenol® and orally infected with *P. gingivalis*. CFU: colony forming units.

Figure 2. Antibacterial effect of Pycnogenol® (PYC) on *P. gingivalis*. Bacterial cells were treated with PBS (○), 1 μg/ml PYC (△), 10 μg/ml PYC (□), 100 μg/ml PYC (●) or 1000 μg/ml PYC (■) for the indicated period.

Figure 3. Morphometric bone levels. The distance from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) was measured at seven buccal sites of the upper jaw per rat (A). Values indicate mean bone loss level ± standard error of the mean. The level for group B, orally infected with *P. gingivalis*, was significantly larger than that for control group A. Administration of Pycnogenol® in addition to bacterial infection significantly inhibited alveolar bone resorption (B). $^{**}p < 0.01$.

Figure 4. The effect of Pycnogenol® (PYC) on cell viability of MC3T3-G2/PA6 cells.

MC3T3-G2/PA6 cells were cultured with various concentrations of PYC for 48 h. Results are expressed as the mean ± SD of (n = 5). $^{**}p < 0.01$, significantly different from the untreated control.

Figure 5. Pycnogenol® (PYC) inhibits osteoclast differentiation of BALB/c mouse bone marrow cells co-cultured with MC3T3-G2/PA6 cells. (A) Mouse bone marrow cells and PA6 cells were co-cultured with 1α,25-dehydroxyvitamin D3 and dexamethasone in the presence or absence of PYC at various concentrations. After incubation for 7 days, co-cultured cells were stained for tartrate-resistant acid phosphatase (TRAP). Arrowheads indicate TRAP-positive multinucleated cells. Bars, 100 μm. (B) TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts. Results were expressed as the mean ± SD of triplicate cultures. $^{**}p < 0.01$.

Figure 6. Pycnogenol® (PYC) inhibits the survival of osteoclasts. (A) Purified osteoclasts with 200 ng/ml receptor activator of NF-κB ligand (RANKL) were treated with PYC extracts for 48 h and stained for tartrate-resistant acid phosphatase (TRAP). Bars, 100 μm. (B) TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts. Results were expressed as the mean ± SD of triplicate

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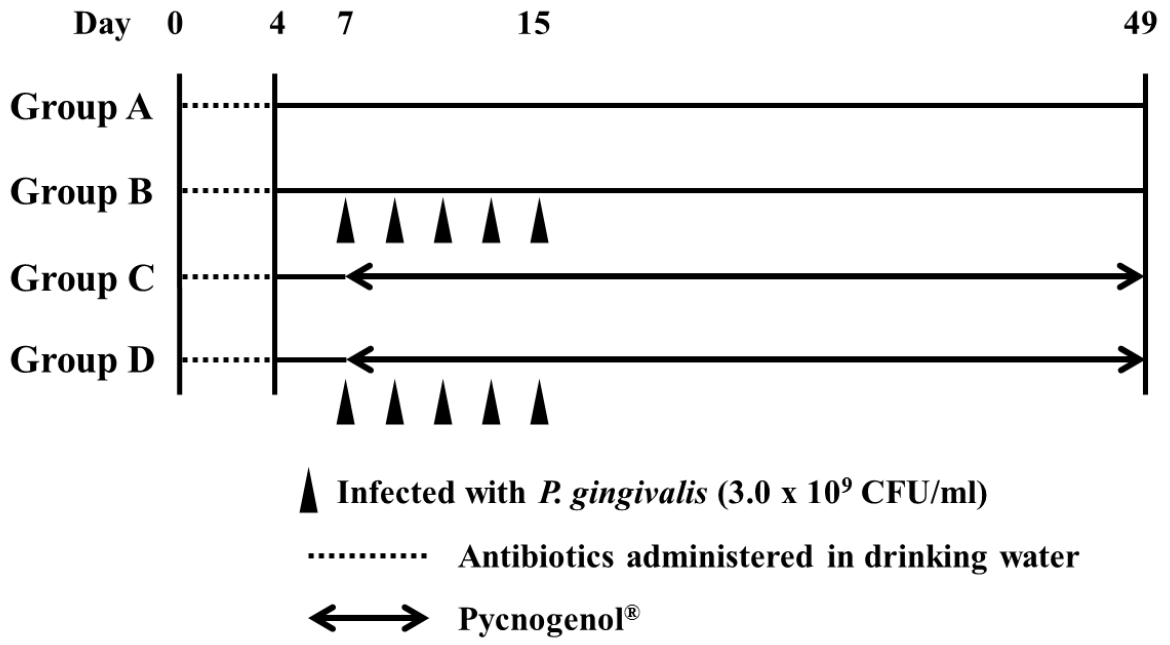
Table 1. Inhibitory effects of Pycnogenol® on *P. gingivalis* invasion

Pycnogenol (μg/ml)	Bacteria adhering and invading[a] (%)	Inhibition (%)	Invasion efficiency[b] (%)	Inhibition (%)
0.0	**5.74 ± 0.67**	**-**	**1.05 ± 0.27**	**-**
1.0	**4.83 ± 0.05**	**15.9**	**0.25 ± 0.05****	**76.2**
10.0	**2.04 ± 0.16****	**64.5**	**0.13 ± 0.03****	**87.6**
100.0	**0.91 ± 0.27****	**84.2**	**0.06 ± 0.0.1****	**94.3**

[a] Defined as the percentage of CFU input that bound to human gingival epithelial cells, which represents both adhering and invading. Values show the means ± standard deviations of triplicate cultures.

[b] Percentage of the *P. gingivalis* inoculum that entry into epithelial cells. Values are the means ± standard deviations of triplicate cultures.

** Significantly different ($p < 0.01$) from the control values without treatment of Pycnogenol.



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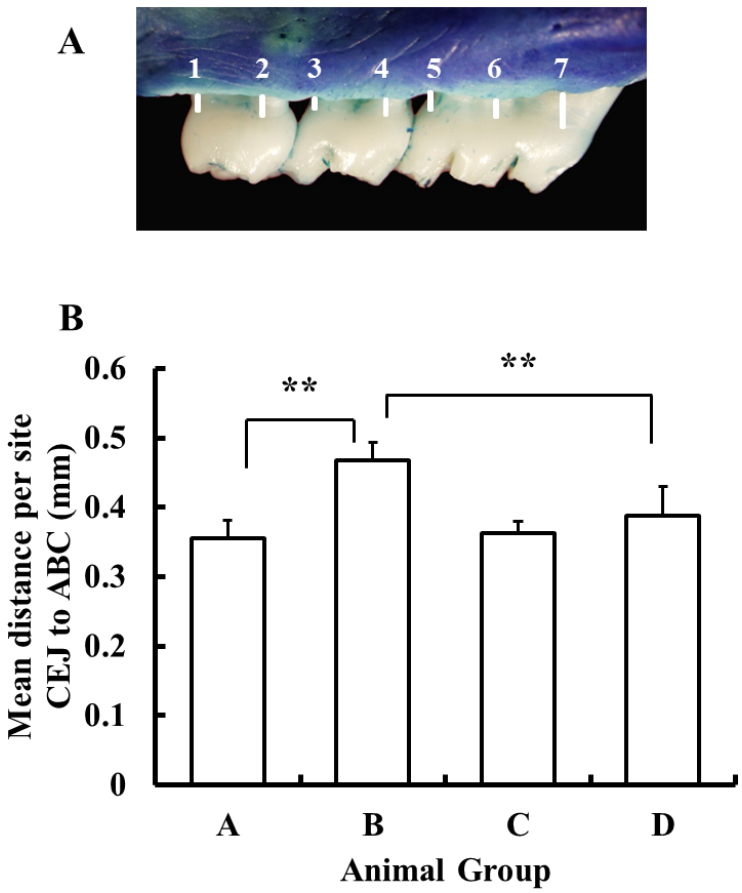

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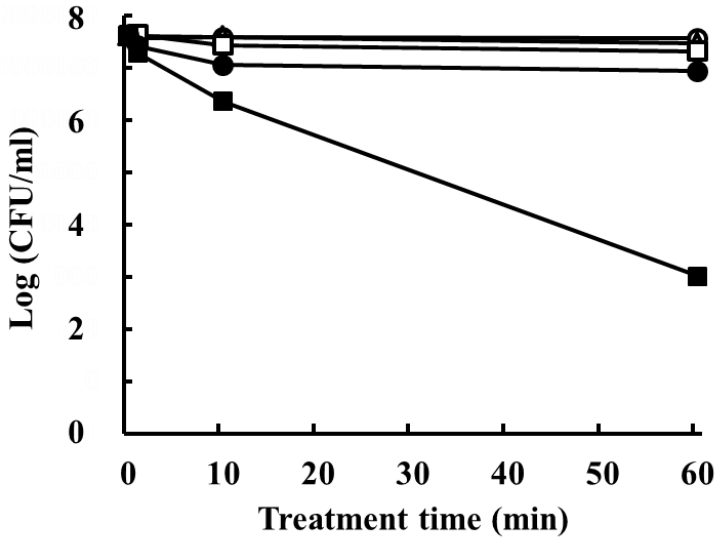

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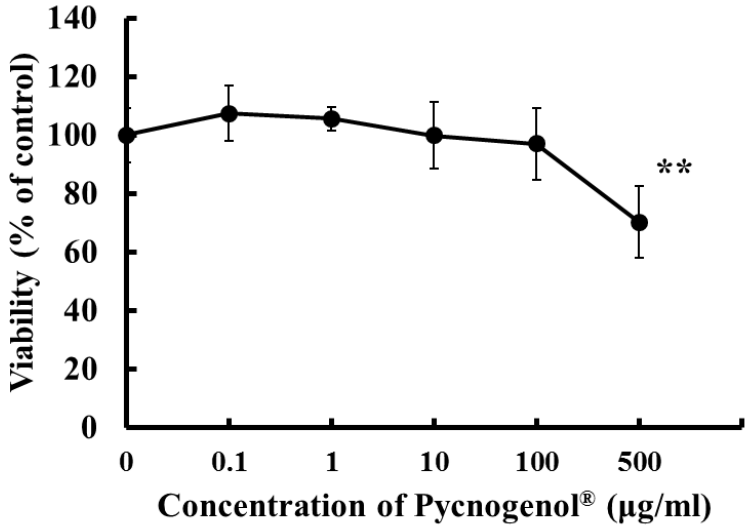

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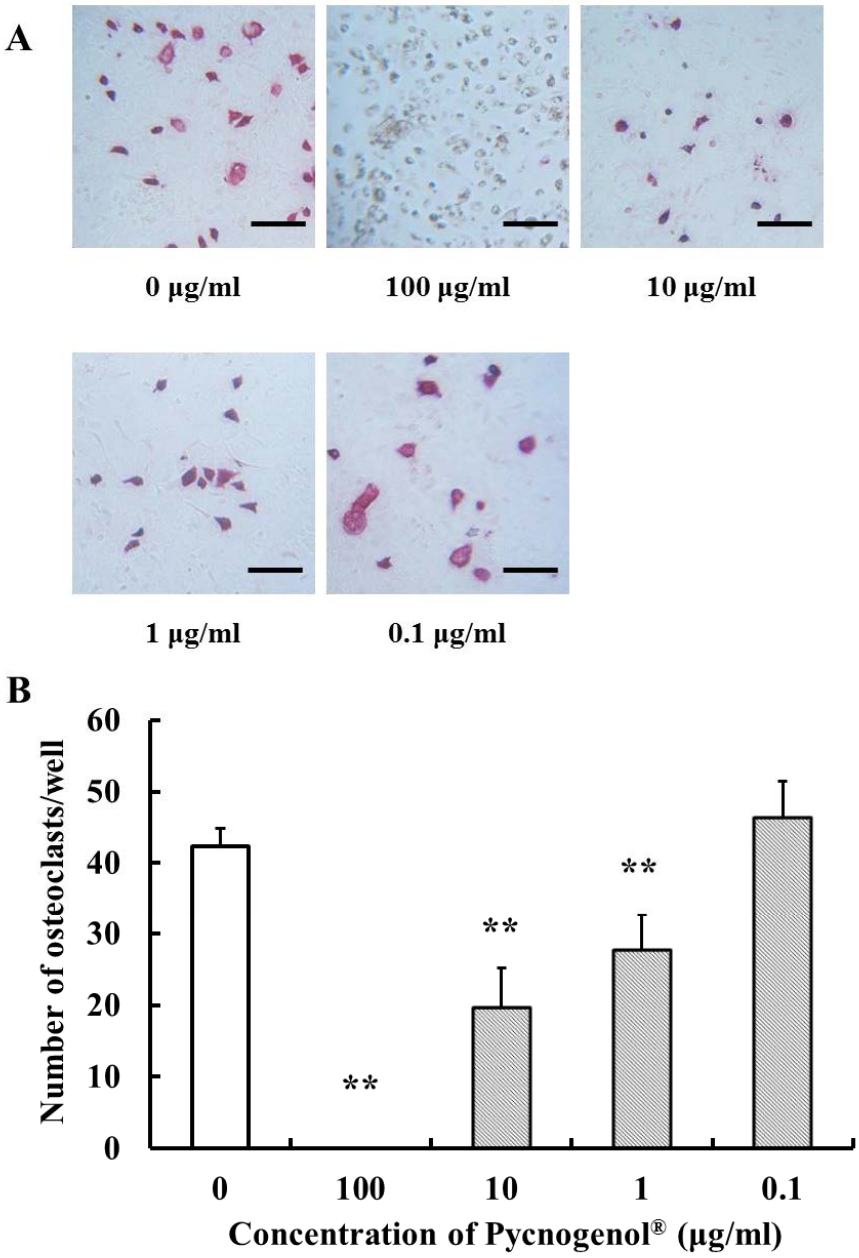

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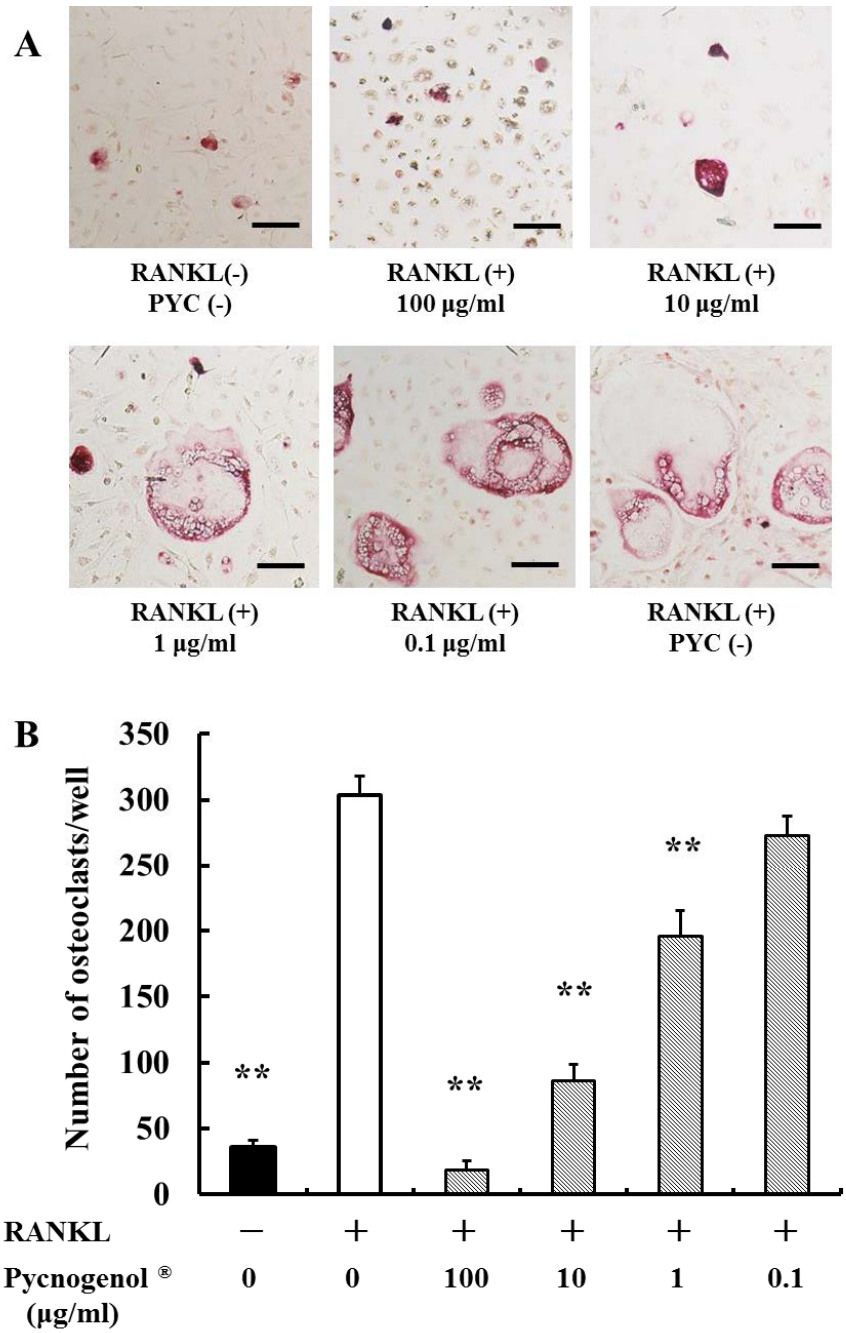

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