Received: 20 April 2018 | Revised: 29 June 2018 | Accepted: 23 July 2018

DOI: 10.1111/jre.12600

ORIGINAL ARTICLE

WILEY Journal of PERIODONTAL RESEARCH

Effects of theaflavins on tissue inflammation and bone resorption on experimental periodontitis in rats

Ya-Hsin Wu[1] | Ryutaro Kuraji[1,2] | Yuji Taya[3] | Hiroshi Ito[1] | Yukihiro Numabe[1]

[1]Department of Periodontology, The Nippon Dental University School of Life Dentistry at Tokyo, Tokyo, Japan

[2]Department of Life Science Dentistry, The Nippon Dental University, Tokyo, Japan

[3]Department of Pathology, The Nippon Dental University School of Life Dentistry at Tokyo, Tokyo, Japan

Correspondence: Ya-Hsin Wu, Department of Periodontology, The Nippon Dental University School of Life Dentistry at Tokyo, 1-9-20 Fujimi, Chiyoda-ku, Tokyo 102-8159, Japan (angela46@tky.ndu.ac.jp).

Funding information
The Nippon Dental University

Background and Objective: Theaflavins (TFs), the major polyphenol in black tea, have the ability to reduce inflammation and bone resorption. The aim of this study was to evaluate the effects of TFs on experimental periodontitis in rats.

Material and Methods: Thirty rats were divided into five groups: Control (glycerol application without ligation), Ligature (glycerol application with ligation), TF1 (1 mg/mL TF application with ligation), TF10 (10 mg/mL TF application with ligation), and TF100 (100 mg/mL TF application with ligation). To induce experimental periodontitis, ligatures were placed around maxillary first molars bilaterally. After ligature placement, 100 μL glycerol or TFs were topically applied to the rats daily, and rats were euthanized 7 days after ligature placement. Micro-computed tomography was used to measure bone resorption in the left side of the maxilla, and quantitative polymerase chain reaction was used to measure the expression of interleukin (*IL*)-*6*, growth-regulated gene product/cytokine-induced neutrophil chemoattractant (*Gro/Cinc-1*, rat equivalent of IL-8), matrix metalloproteinase-9 (*Mmp-9*), receptor activator of nuclear factor-kappa B ligand (*Rankl*), osteoprotegerin (*Opg*), and the *Rankl/Opg* ratio in gingival tissue. With tissue from the right side of the maxilla, hematoxylin and eosin staining was used for histological analysis, immunohistochemical staining for leukocyte common antigen (CD45) was used to assess inflammation, and tartrate-resistant acid phosphatase (TRAP) staining was used to observe the number of osteoclasts.

Results: The TF10 and TF100 groups, but not the TF1 group, had significant inhibition of alveolar bone loss, reduction in inflammatory cell infiltration in the periodontium, and significantly reduced numbers of CD45-positive cells and TRAP-positive osteoclasts compared with the Ligature group. Correspondingly, the TF10 and TF100 groups had significantly downregulated gene expression of *IL-6*, *Gro/Cinc-1*(*IL-8*), *Mmp-9*, and *Rankl*, but not of *Opg*. Consequently, *Rankl/Opg* expression was significantly increased in the Ligation group but was attenuated in the TF10 and TF100 groups.

Conclusion: The results of this study suggest that topical application of TFs may reduce inflammation and bone resorption in experimental periodontitis. Therefore, TFs have therapeutic potential in the treatment of periodontal disease.

KEYWORDS

cytokines, experimental periodontitis, periodontal disease, theaflavin

1 | INTRODUCTION

Periodontal disease is one of the most common chronic diseases in humans and is the main cause of tooth loss.[1] Periodontal bacteria play a critical role in the primary etiology of this disease; however, the advanced progression of periodontal tissue destruction relies on the host immune response.[2] Representative key proinflammatory cytokines and chemokines in the immune system are interleukin (IL)-6 and IL-8, respectively. These mediators are thought to be associated with the progression of periodontal disease,[3] as they promote leukocyte extravasation and induce the release and activation of matrix metalloproteinases (MMPs), especially MMP-9.[4,5] MMP-9 plays an important role in the progression of periodontitis via removal of the collagenous layer from the bone surface, which helps initiate osteoclastogenesis.[6]

Other critical factors that mediate osteoclastogenesis are receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin (OPG). RANKL induces osteoclast formation and activity by interacting with its receptor, RANK, which is expressed on the surface of osteoclasts, whereas OPG competitively binds to RANK to abolish this effect.[7] Thus, the RANKL/OPG ratio in osteoclastogenesis is a major determinant of bone resorption. In inflamed tissue, IL-6 stimulates the secretion of RANKL and decreases OPG expression,[8,9] which represents the activation of osteoclasts and results in bone loss.[10]

The conventional treatment for periodontitis relies on the mechanical removal of bacterial plaque from the periodontal pockets by scaling and root planing.[11] Recently, a new treatment strategy based on modulating the inflammatory response to periodontal disease has been advocated.[12] Natural plants have abundant antiinflammatory materials, many of which are pharmacologically safe.[13] Therefore, plant polyphenols have been proposed as promising compounds for adjunctive periodontal therapy.[14-16]

Black tea, derived from the leaves of *Camellia sinensis* similar to green tea, accounts for 78% of the world's tea consumption.[17] The major flavonoid in green tea is catechin. Correspondingly, theaflavins (TFs) are the main polyphenol present in black tea, formed by enzyme-catalyzed oxidative dimerization of catechins during fermentation of green tea to black tea. TFs comprise a mixture of theaflavin (TF-1), theaflavin-3-gallate (TF-2a), theaflavin-3′-gallate (TF-2b), and theaflavin-3,3′-digallate (TF-3).[18] Similar to catechins, previous studies have shown that TFs have many positive effects on human health including antioxidant,[19] antiinflammatory,[20,21] and antitumor effects.[22] Furthermore, compared with catechins, TF-3 more effectively blocked lipopolysaccharide (LPS)-induced nuclear factor-kappa B (NF-κB) activation by inhibiting IkappaB kinase activity in murine macrophages[23] and more effectively inhibited osteoclast formation.[24]

In dental research studies, TFs reduced IL-8 secretion in LPS-stimulated oral epithelial cells.[25] In addition, TF-3 inhibited tumor necrosis factor receptor superfamily 14-induced IL-6 production in human gingival fibroblasts.[26] Recently, it was reported that TFs attenuated the secretion of IL-6, chemokine ligand 8 (CXCL-8 or IL-8), and MMP-9 in macrophages stimulated by *Porphyromonas gingivalis*.[27]

To date, many studies have shown the potential treatment effects of catechins on periodontitis in vitro and in vivo.[28,29] Recently, locally delivered green tea extract gel has been used as an adjunct to nonsurgical periodontal treatment.[30] However, few studies have shown the potential effects of TFs in the control of periodontal disease, and these studies were limited to in vitro experiments.[27] Thus, both the in vivo effects of TFs on individual inflammatory responses and the possibility for clinical application are poorly understood.

Therefore, the aim of this study was to evaluate the therapeutic effects of TFs on ligature-induced experimental periodontitis in rats.

2 | MATERIAL AND METHODS

2.1 | Animals and experimental design

All experimental procedures were approved by the Animal Experiments Committee of The Nippon Dental University School of Life Dentistry at Tokyo (No. 15-23-3). A total of thirty 8-week-old male Wistar rats weighing 180-200 g (Clea Japan, Osaka, Japan) were included in the experiment. All rats were raised in cages at 23°C and 50% humidity under a 12-hours light-dark cycle, and received water and food ad libitum. Animals were housed in this environment for 1 week before the start of the experiments. They were randomly divided into the following five groups (n = 6): (a) Control, glycerol application without ligation; (b) Ligature, glycerol application with ligation; (c) TF1, 1 mg/mL TF application with ligation; (d) TF10, 10 mg/mL TF application with ligation; and (e) TF100, 100 mg/mL TF application with ligation (Figure 1).

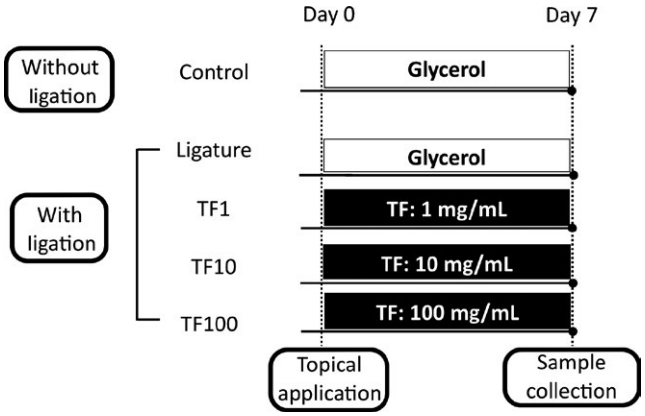

FIGURE 1 Experimental groups. Control, glycerol application without ligation; Ligature, glycerol application with ligation; TF1, 1 mg/mL TF application with ligation; TF10, 10 mg/mL TF application with ligation; and TF100, 100 mg/mL TF application with ligation

2.2 | Ligature placement around the cervical region of the maxilla molar

To induce experimental periodontitis, ligature placement around the molar tooth was performed according to the method of Kuraji et al.[31] After the rats were anesthetized with sodium pentobarbital (50 mg/kg), a sterile 3-0 silk suture was placed around each cervix of the first molar (M1) on bilateral sides in the maxilla in the open mouth state using Hashimoto's Gag (Nonaka Rikaki, Tokyo, Japan). The knot of the suture was fixed with composite resin to the mesial site of the M1. The M1 in the Control group did not undergo ligature placement.

2.3 | TFs preparation and treatment

Theaflavins were obtained from Yaizu Suisankagaku Industry (Shizuoka, Japan), and the constituent molecular species of TFs were 5% TF1, 19% TF-2a, 7% TF-2b, and 49% TF-3. TF concentrations of 1, 10, or 100 mg/mL were dissolved in 80% glycerol. After ligature placement, 50 μL TF solution was topically applied to rats in the TF groups around each M1 on the bilateral side once a day for 1 week. In the Control and Ligature groups, only 80% glycerol was topically applied to the M1 in the same manner as in the TF groups.

2.4 | Sample collection

Rats were euthanized under general anesthesia 7 days after ligature placement. Gingival tissues on the left side of the M1 were immediately removed and stored in Allprotect Tissue Reagent (Qiagen, Valencia, CA) at −20°C. The maxilla resected from the rat was fixed in 4% paraformaldehyde for 1 day and then transferred to 70% ethanol solution.

2.5 | Micro-computed tomography analysis

The fixed specimens from the left side of the maxilla were scanned using a micro-computed tomography (micro-CT) imaging system (ScanXmate-D100SS270; Comscan, Kanagawa, Japan). Measurement conditions for micro-CT were as follows: tube voltage, 65 kV; electrical current, 80 μA; image pixel size, 1024 × 1024; and slice thickness, 15 μm. After scanning, 3-dimensional (3D) images were reconstructed using TRI/3D-BON version 64 software (Ratoc System Engineering, Tokyo, Japan) in three regions (mesial, central, and distal sites) of the M1 (Figure 2A), the distance from the palatal cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) was measured as previously described.[32] The average of the three distances was defined as the representative value for each rat. The volumetric measurement of furcation was performed after the selection of a region of interest (ROI), which was selected according to the following standardized dimensions and anatomical landmarks: (a) a line 0.5 mm apical to the CEJ of the first molar as the coronal limit extended vertically 1 mm to the root apical, (b) a line 3 mm with the mesial-distal dimension from the mesial CEJ of the first molar, and (c) a line 2.5 mm with the buccal-palatal dimension (Figure 2B). The microstructural parameter, bone volume fraction (BV/TV), which means the percentage of the ROI filled with bone volume, was measured using TRI/3D-BON software (Ratoc System Engineering).

2.6 | Histopathological evaluation

The right side of each maxilla was decalcified with 10% ethylenediaminetetraacetic acid solution (pH 7.0) (Muto Pure Chemicals Co., Tokyo, Japan) for 4 weeks at 4°C. The tissue blocks were embedded in paraffin, and serial bucco-lingual sections (5 μm thick) of each maxilla were stained with hematoxylin and eosin (H&E).

To confirm the presence of inflammatory cells, the pan-leukocyte marker CD45 was stained by immunohistochemistry. To this end, the sections were deparaffinized, and antigens were retrieved by microwaving in 10 mmol/L Tris-EDTA buffer (pH 9.0) for 10 minutes at 90°C. Then, the sections were incubated with Dako protein block solution (Dako, Carpinteria, CA) for 20 minutes to block nonspecific staining. Immediately afterward, the sections were incubated overnight at 4°C with rabbit anti-CD45 primary antibody (1:200, ab10558; Abcam, Cambridge, MA). After washing with

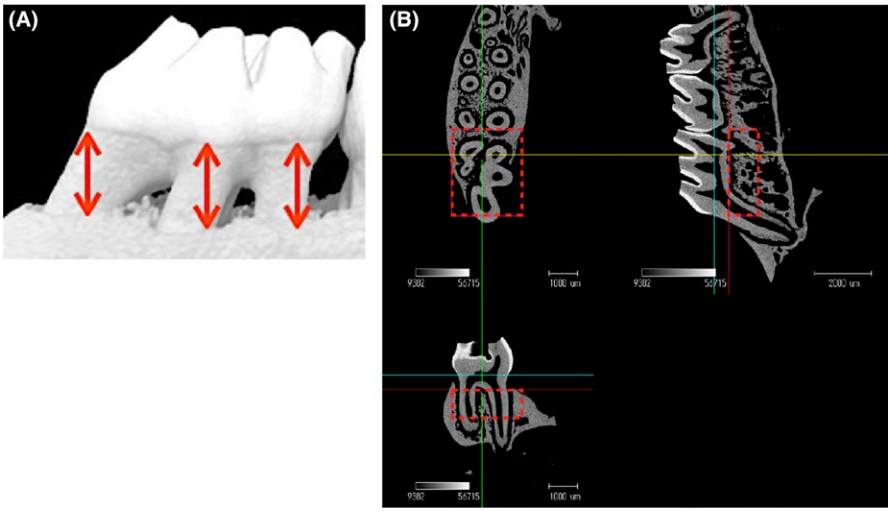

FIGURE 2 Reconstructed 3D micro-CT images. A, The arrows show the distance from the palatal CEJ to the ABC as a marker of alveolar bone height; the length of three areas (mesial, central, and distal sites) of the maxillary M1 was measured, and the mean value was determined as experimental data. B, A cube region was selected as the ROI to evaluate the ratio of the bone volume fraction (BV/TV)

phosphate-buffered saline, the sections were incubated for 1 hour with goat anti-rabbit IgG as fluorescein secondary antibody (1:50, Alexa Fluor 488; Abcam). Finally, the slides were mounted with SlowFade Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA).

To identify osteoclasts, tartrate-resistant acid phosphatase (TRAP) activity was detected using the TRAP/ALP Kit (Wako Pure Chemical Industries Co., Osaka, Japan). TRAP staining solution was added to the deparaffinized specimens and reacted for 30 minutes at room temperature with close monitoring until the bright red staining, representing osteoclast activity, was observed. After washing with distilled water, tissues were counterstained with the nuclear staining solution included in the kit (blue color) and mounted with Vectamount (Vector Laboratories, Burlingame, CA). H&E and TRAP histological observations were performed from photographs captured under an optical microscope (Nikon Eclipse 50i POL microscope; Nikon Instruments, Tokyo, Japan). Digital images of immunohistochemistry were recorded by virtual microscopy (NanoZoomer HT; Hamamatsu Photonics, Hamamatsu, Japan).

2.7 | Quantification of CD45- and TRAP-positive cells

The number of CD45-positive cells was calculated according to the method of Yoshinaga et al[28] with some modifications. Four square fields (100 × 100 μm) of connective tissue adjacent to the junctional epithelium (JE) were selected from each section (Figure 3). Three tissue sections per rat specimen (n = 5 per group) were measured, and three counted outcomes were averaged for each section. Data are expressed as the mean number of cells per 1.0 mm^2 of connective tissue.

The analysis of osteoclast numbers was conducted according to the method of Sanbe et al.[33] The criteria for determination of osteoclasts were TRAP-positive multinucleated cells. The numbers of TRAP-positive multinucleated cells on the linear surface of alveolar bone were counted. The measurement regions were performed based on location as shown in Figure 3, and included the segment from the top of the alveolar bone crest to the starting point of the curve as the horizontal segment (Line X), and from the top of the alveolar bone crest to the apex along the periodontal ligament as the vertical segment (Line Y). The numbers of TRAP-positive multinucleated cells in two segments were divided by the line of the alveolar bone surface. Three tissue sections per rat specimen (n = 6 per group) were measured, and three counted outcomes were averaged for each section. Data are expressed as the mean number of cells per 1.0 mm of alveolar bone.

2.8 | RNA extraction and quantitative polymerase chain reaction

Total RNA from each gingival sample (n = 6 per group) was extracted using the Maxwell® RSC simplyRNA Kits (Promega, Madison, WI). The purity and quantity of the RNA were evaluated using the NanoVue Plus spectrophotometer (Biochrom Ltd., Cambridge, UK). Total RNA (1 μg) was synthesized into cDNA using the SuperScript VILO Master Mix (11755050; Invitrogen). Then, the cDNA samples were stored at −20°C. The relative gene expression was measured by quantitative polymerase chain reaction (qPCR) using the following TaqMan primers and probes (TaqMan Gene Expression Assays; Applied Biosystems, Carlsbad, CA): *IL-6* (Rn01410330_m1), *Mmp-9* (Rn00579162_m1), *Rankl* (Rn00589289_m1), *Opg* (Rn00563499_m1), and the growth-regulated gene product/cytokine-induced neutrophil chemoattractant (*Gro/Cinc-1*, rat equivalent of IL-8; Rn00578225_m1). It is worth noting that rats do not have an ortholog of the human IL-8 peptide, so *Gro/Cinc-1*, a rat chemokine with structural and functional homology to human IL-8, was used in this study.[34] Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*; Rn01775763_g1) was used as a housekeeping gene to normalize the amount of mRNA present in each reaction. The reactions were conducted in a final reaction volume of 20 μL that included the TaqMan Fast Advanced Master Mix (Applied Biosystems), cDNA template (corresponding to 10 ng/μL cDNA), primers, and probes. The optimized thermal cycling conditions were as follows: 20 minutes at 95°C, followed by 40 cycles per 1 minute at 95°C, and 20 minutes

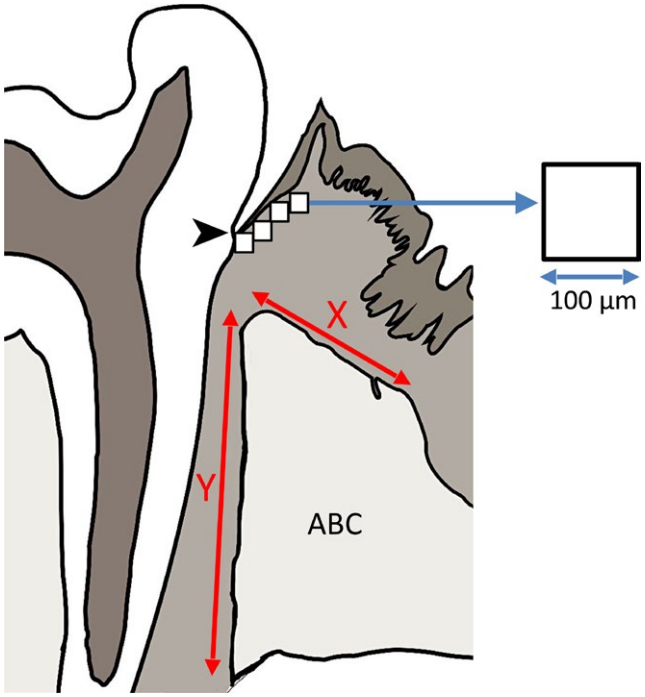

FIGURE 3 Schematic illustration of the target regions of CD45-positive and TRAP-positive multinucleated cells of rat periodontal tissue. The four square fields (100 × 100 μm) of connective tissue adjacent to the JE indicate target areas that were used to count CD45-positive cells. TRAP-positive multinucleated cells were calculated on the linear surface of the alveolar bone. The segment from the top of the alveolar bone crest to the starting point of the curve was designated as the horizontal segment (Line X), and that from the top of the alveolar bone crest to the apex along the periodontal ligament was designated as the vertical segment (Line Y). Black arrow, cemento-enamel junction. ABC, alveolar bone crest

at 60°C. To compare the expression levels among different samples, the relative expression level of the genes was calculated by the comparative CT (ΔΔCT) method using StepOnePlus software (Applied Biosystems).

2.9 | Statistical analysis

To calculate the sample size in each group, 80% power to recognize a significant difference among the groups with a 95% confidence interval (a = 0.05) was determined using G*Power 3.1.9.2 software (Heinrich-Heine-University Dusseldorf, Düsseldorf, Germany). The CEJ-ABC distance in our pilot study was considered. Based on these data, the required minimum sample size of rats was determined as 6.

Normal distribution patterns for respective data were demonstrated by the Kolmogorov-Smirnov test. One-way analysis of variance and the post hoc Tukey test were used to compare the five groups. All statistical analyses were performed with SPSS software (version 15.0J; SPSS, Chicago, IL). P values less than 0.05 were considered statistically significant. The results are expressed as mean ± standard deviation or percentage.

3 | RESULTS

3.1 | Micro-CT analysis of bone resorption

The reconstructed 3D images of the five groups are shown in Figure 4A. The CEJ-ABC distances in the Ligature group and all TF groups increased compared with those in the Control group. However, bony breakdown and furcation involvement were less severe in the TF10 and TF100 groups than in the Ligature and TF1 groups. Measurement of the CEJ-ABC distance revealed that the Ligature group had the most severe bone resorption of all groups (Figure 4B). Only the TF10 and TF100 groups had a significant distance decrease compared with the Ligature group by 10% and 12.6%, respectively (Figure 4B; P < 0.001). In the bone volume fraction analysis, the BV/TV ratio (Figure 4C) was remarkably decreased in the Ligature group (P < 0.001), but the decreased ratio was significantly attenuated in the TF10 and TF100 groups.

There were no significant differences in the CEJ-ABC distance and BV/TV ratio between the TF10 and TF100 groups.

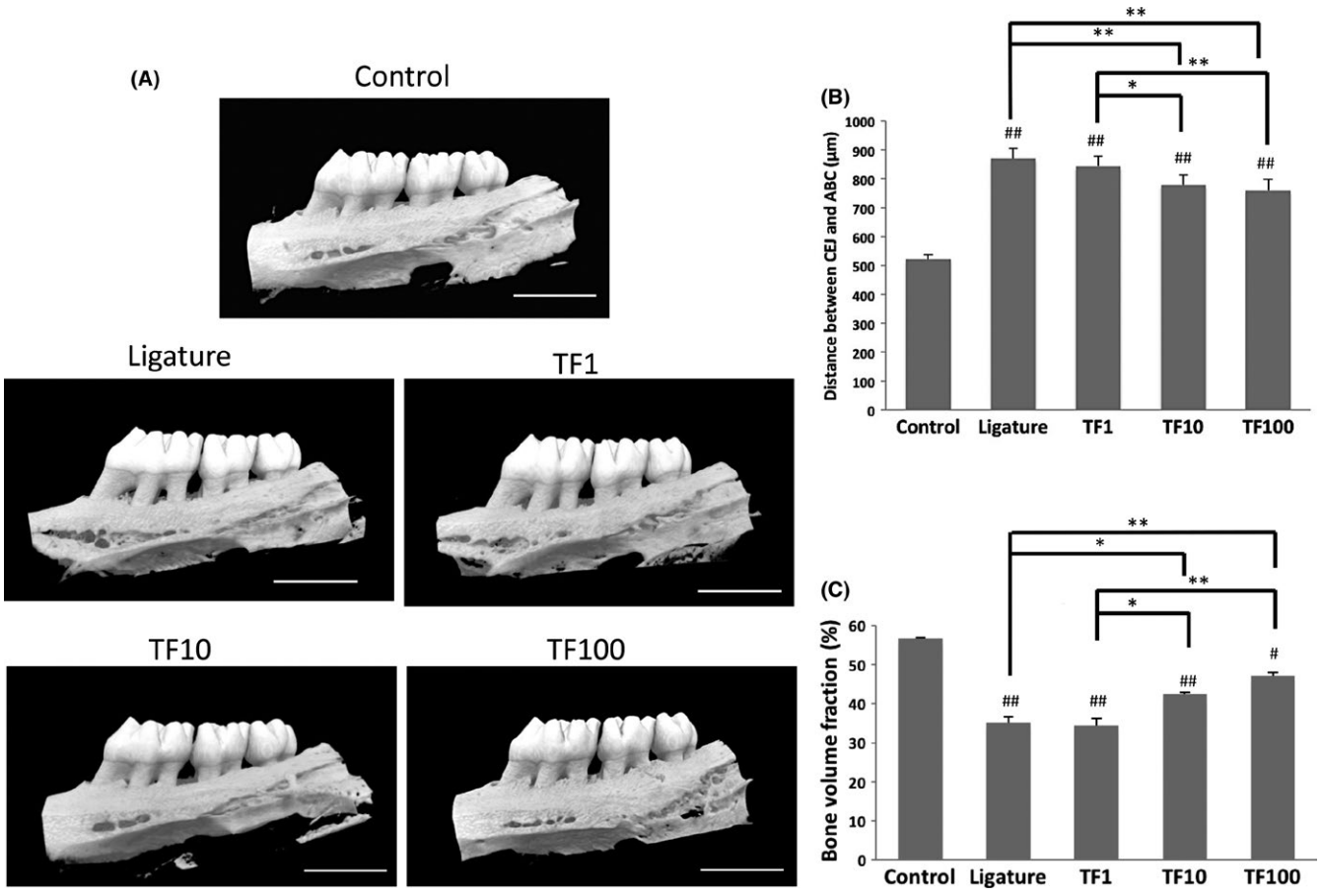

FIGURE 4 Effects of TFs on alveolar bone loss. A, Reconstructed 3D micro-CT images show the palatal view of the maxilla (scale bar = 3000 μm). B, The CEJ-ABC distance data are expressed as mean ± SD. C, The BV/TV ratio in the interradicular regions of the first molars is expressed as mean ± SD. $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.001 compared with the Control group; $^{*}P$ < 0.05, $^{**}P$ < 0.001 compared with the Ligature group. Control, glycerol application without ligation; Ligature, glycerol application with ligation; TF1, 1 mg/mL TF application with ligation; TF10, 10 mg/mL TF application with ligation; and TF100, 100 mg/mL TF application with ligation

3.2 | Histological and quantitative analysis of inflammatory infiltration

H&E staining of periodontal tissue exhibited a JE that was situated in the CEJ in the Control group (Figure 5A,F). However, compared with the Control group, the periodontal tissue in all of the other groups with ligature placement had pouchlike periodontal pockets, and apical migration of the JE along the tooth surface was apparent on the exposed cementum (Figure 5B-J). The thin and discontinuous JE were observed in the Ligature and TF1 groups (Figure 5G,H), whereas thick and continuous JE were observed in the TF10 and TF100 groups (Figure 5I,J).

To assess inflammatory infiltration, H&E staining and immunohistochemical staining for CD45 (also known as leukocyte common antigen) were performed. Very few inflammatory cells were present around the connective tissue in the Control group (Figures 5A,F and 6A), and the number of CD45-positive cells was 3.1 ± 0.3 cells/mm^2 (Table 1). Compared with the Control group, all other groups with ligature placement had significantly increased numbers of CD45-positive cells ($P < 0.001$). In the Ligature group (Figures 5B,G and 6B) and the TF1 group (Figures 5C,H and 6C), inflammatory cells infiltrated into the lamina propria, and abundant numbers of CD45-positive cells were observed. The TF10 (Figures 5D,I and 6D) and TF100 (Figures 5E,J and 6E) groups showed a decrease in both the number of inflammatory cells and apical migration of the JE compared with the Ligature and TF1 groups. Consistent with this result, the numbers of CD45-positive cells were significantly decreased in the TF10 and TF100 groups compared with the Ligature and TF1 groups, but there was no significant difference in cell number between the TF10 and TF100 groups (Table 1).

3.3 | Histological and quantitative analysis of osteoclast numbers

TRAP staining of the periodontal tissue showed that only a few osteoclasts were present on the Line X and Y segments of the Control group (Figure 7A,F), and quantification of the Line X + Y segments was 1.8 ± 0.6 cells/mm (Table 2). Numerous TRAP-positive osteoclasts were observed in the Ligature (Figure 7B,G) and TF1 (Figure 7C,H) groups, and quantification of the Line X + Y segments was 11.7 ± 1.3 and 9.3 ± 2.4 cells/mm, respectively (Table 2). However, the TRAP-positive cells were evidently reduced in both TF10 (Figure 7D,I) and TF100 (Figure 7E,J) groups.

To identify correlating morphological changes, we evaluated the number of TRAP-positive cells with Line X and Line Y segments separately. On the Line X segment, the Ligature group showed a significantly increased number of TRAP-positive cells on the alveolar bone surface compared with the Control group (Ligature, 18.0 ± 4.9 cells/mm; $P < 0.001$) (Table 2). This result was similar to the TF1 group compared with the Control group (TF1, 12.4 ± 5.0 cells/mm; $P < 0.001$),

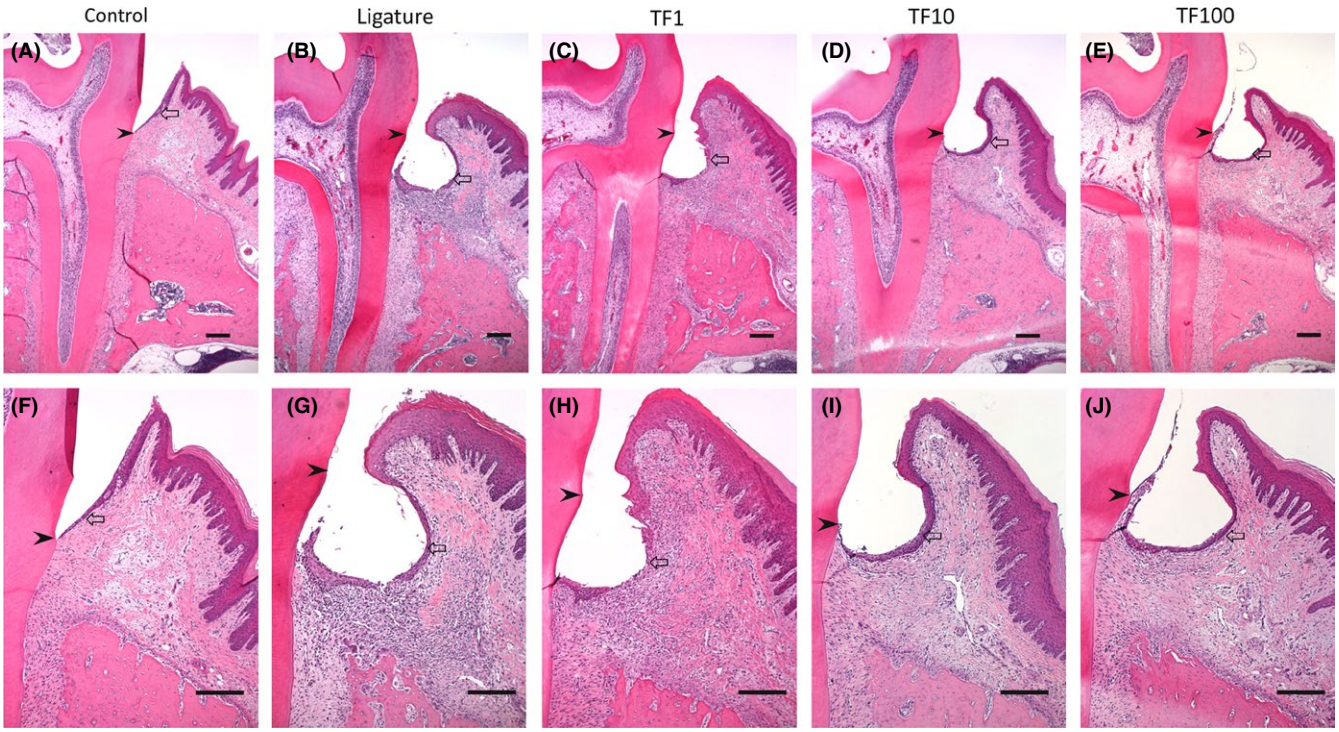

FIGURE 5 Histological evaluation of inflammatory infiltration by H&E staining. H&E staining of specimens from the Control group (A, F), Ligature group (B, G), TF1 group (C, H), TF10 group (D, I), and TF100 group (E, J). Higher magnification micrographs show that the TF10 (I) and TF100 (J) groups had reduced inflammatory cell infiltration and less cementum exposed than the Ligature (G) and TF1 (H) groups. Control, glycerol application without ligation; Ligature, glycerol application with ligation; TF1, 1 mg/mL TF application with ligation; TF10, 10 mg/mL TF application with ligation; and TF100, 100 mg/mL TF application with ligation. Black arrows, cemento-enamel junction (CEJ); white arrows, junctional epithelium (JE). Scale bar = 200 μm

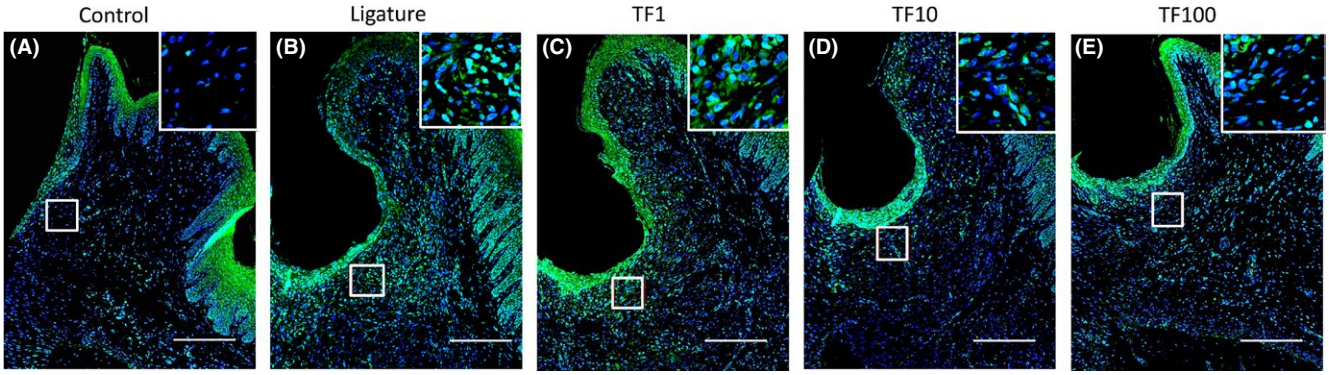

FIGURE 6 Histological evaluation of inflammatory infiltration by immunohistochemical staining. CD45 (green) staining of specimens from the Control group (A), Ligature group (B), TF1 group (C), TF10 group (D), and TF100 group (E). Nuclear counterstaining by DAPI (blue) in fluorescent images. Higher magnification micrographs in the upper right corner show that the TF10 (D) and TF100 (E) groups had reduced CD45-positive cell infiltration compared with the Ligature (B) and TF1 (C) groups. Control, glycerol application without ligation; Ligature, glycerol application with ligation; TF1, 1 mg/mL TF application with ligation; TF10, 10 mg/mL TF application with ligation; and TF100, 100 mg/mL TF application with ligation. Scale bar = 200 μm

TABLE 1 Quantitative analysis of CD45-positive cells (mean ± SD)

Number of CD45-positive cells/ unit square (cells/mm^2)	Control	Ligature	TF1	TF10	TF100
Cell numbers × 10^2	3.1 ± 0.3	23.8 ± 2.6$^{\#\#}$	20.9 ± 3.1$^{\#\#}$	15.1 ± 1.6$^{\#\#,**,¶}$	12.8 ± 1.3$^{\#\#,**,¶¶}$

Control, glycerol application without ligation; Ligature, glycerol application with ligation; TF1, 1 mg/mL TF application with ligation; TF10, 10 mg/mL TF application with ligation; and TF100, 100 mg/mL TF application with ligation.
$^{\#\#}P < 0.001$ was considered significantly different compared with the Control group. $^{**}P < 0.001$ was considered significantly different compared with the Ligature group. $^{¶}P < 0.05$, $^{¶¶}P < 0.001$ was considered significantly different compared with the TF1 group.

but there was no significant difference compared with the Ligature group. In contrast, the numbers of TRAP-positive osteoclasts in the TF10 and TF100 groups were significantly lower than those in the Ligature group (TF10, 6.1 ± 2.4 cells/mm; TF100; 7.1 ± 5.2 cells/mm; $P < 0.001$), but no significant difference was observed between the TF10 and TF100 groups. On the Line Y segment, compared with the Control group, all other groups with ligature placement had significantly increased numbers of TRAP-positive cells ($P < 0.05$), with the exception of the TF100 group (Table 2). However, the difference among all groups with ligature placement was not statistically significant ($P > 0.05$).

3.4 | mRNA expression of inflammatory mediators in gingival tissue

To observe the expression of inflammatory mediator-related genes, the mRNA levels in rat gingival tissues were evaluated by qPCR (Figure 8). A significant increase in the mRNA expression of *IL-6*, *Mmp-9* ($P < 0.001$), and *Gro/Cinc-1*(*IL-8*) ($P < 0.05$) was observed in the Ligature group compared with the Control group. The TF1 group showed a tendency to repress the expression of inflammatory mediators, and only the mRNA expression of *IL-6* was significantly lower than that in the Ligature group (Figure 8A; $P < 0.05$). On the other hand, the TF10 and TF100 groups exhibited significantly attenuated expression of *IL-6* ($P < 0.001$), *Mmp-9*, and *Gro/Cinc-1* ($P < 0.05$) relative to the Ligature group (Figure 8A-C). *Rankl* expression was markedly higher in the Ligature group compared with the Control group (Figure 8D; $P < 0.001$). As expected, the TF10 and TF100 groups had significant inhibition of ligature-induced *Rankl* expression (Figure 8D; $P < 0.05$). Conversely, compared with the Ligature group, there was no significant difference in *Opg* expression among all groups (Figure 8E). Consequently, the *Rankl/Opg* ratio was remarkably increased in the Ligature group ($P < 0.001$), whereas the elevated ratio was significantly attenuated in the TF10 and TF100 groups (Figure 8F; $P < 0.05$).

4 | DISCUSSION

Ligature-induced experimental periodontitis has been a reliable and widely used technique for decades.[35] After ligature placement around the cervical region of the M1, a large amount of plaque and sulcular epithelium ulceration is induced.[36] This action causes the host immune response that leads to inflammatory cell infiltration into the gingival tissue and bone resorption. In this study, numerous inflammatory cells infiltrated into the lamina propria, and JE apical migration was observed histologically in the Ligature group, 7 days after ligature placement. In addition, micro-CT results showed an increased CEJ-ABC distance in the Ligature group compared with the Control group. These results were in accordance with those by

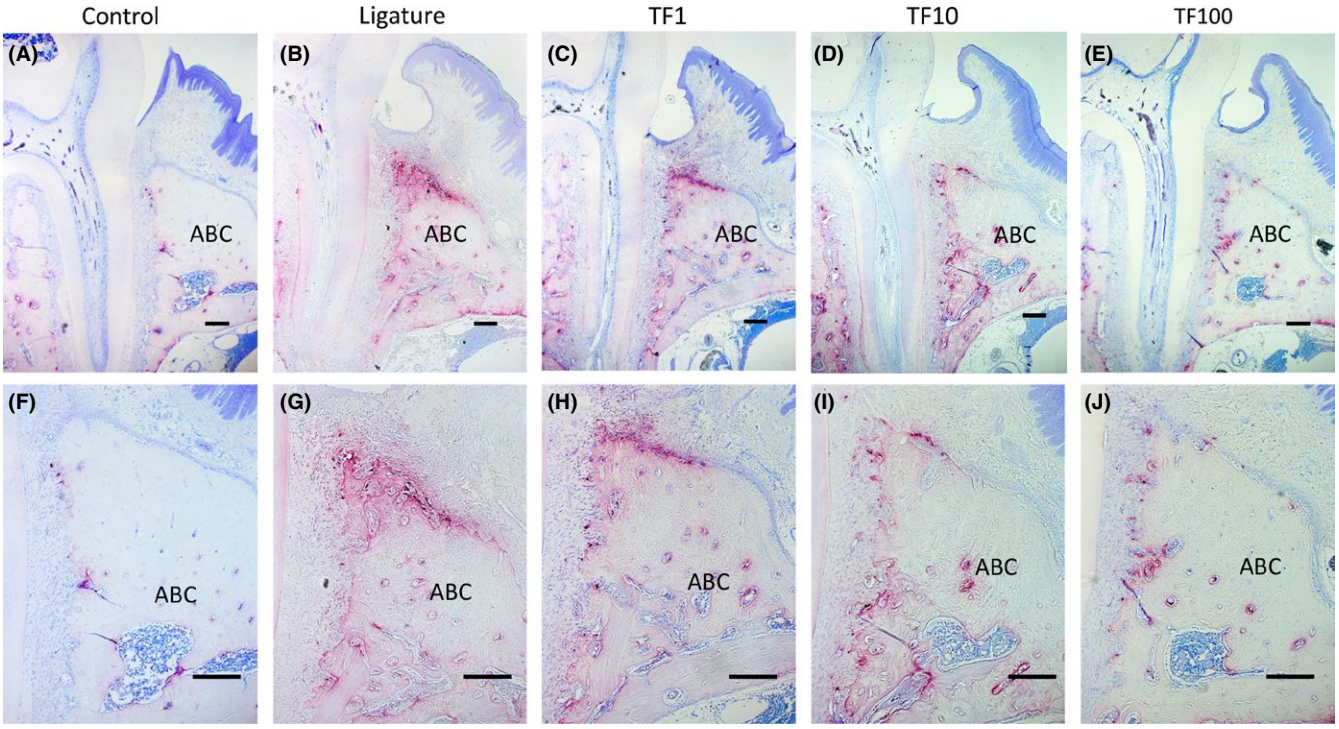

FIGURE 7 Histological evaluation of osteoclasts. Osteoclasts were stained red by TRAP staining. Specimens from the Control group (A, F), Ligature group (B, G), TF1 group (C, H), TF10 group (D, I), and TF100 group (E, J) are shown. Higher magnification micrographs show that the Control group (F) had few TRAP-positive cells on the Line Y segment. The TF10 (I) and TF100 (J) groups had fewer TRAP-positive cells than the Ligature (G) and TF1 (H) groups, especially on the Line X segment. Control, glycerol application without ligation; Ligature, glycerol application with ligation; TF1, 1 mg/mL TF application with ligation; TF10, 10 mg/mL TF application with ligation; and TF100, 100 mg/mL TF application with ligation. ABC, alveolar bone crest. Scale bar = 200 μm

Liu et al[37] who found large gingival inflammation and alveolar bone resorption 7 days after ligation, which stabilized after 11 days. These results indicate that experimental periodontitis was successfully established in this study.

As aforementioned, inhibition of inflammatory cell recruitment and proinflammatory mediator production is considered an important step to target in the treatment of periodontitis. Recently, polyphenols were shown to modulate the host response, and their low toxicity has caused them to receive much attention.[14] TFs, the major polyphenol in black tea, have many well-documented antiinflammatory and antiosteoclastogenesis effects. Therefore, this study was designed to determine whether TFs can inhibit inflammation and bone loss in a ligature-induced experimental model. To the best of our knowledge, this is the first study to evaluate the effects of TFs on ligature-induced experimental periodontitis in rats.

Several studies have investigated the pharmacological effects of TFs. For example, in a previous study, oral administration of 50-200 mg/kg TFs for 5 weeks led to the dose-dependent attenuation of cadmium-induced testicular toxicity in male rats.[38] Numerous studies have shown that TFs have poor systemic bioavailability by oral administration. In a human study, after consumption of 700 mg of TFs, the maximum concentration of TFs in plasma was only 1.0 ng/mL.[39] Recently, Pereira-Pereira-Caro et al[40] found that TFs were not absorbed in either the upper or lower gastrointestinal tract. Therefore, in this study, we decided to administer TFs topically, instead of by oral administration.

TABLE 2 Quantitative analysis of TRAP-positive osteoclasts (mean ± SD)

Number of cells/bone surface length (cells/mm)	Control	Ligature	TF1	TF10	TF100
Line X + Y segment	1.8 ± 0.6	11.7 ± 1.3$^{\#\#}$	9.3 ± 2.4$^{\#\#}$	7.0 ± 2.0$^{\#\#,*}$	7.2 ± 2.4$^{\#\#,*}$
Line X segment	0.3 ± 0.5	18.0 ± 4.9$^{\#\#}$	12.4 ± 5.0$^{\#\#}$	6.1 ± 2.4$^{\#,**}$	7.1 ± 5.2$^{\#,**}$
Line Y segment	2.8 ± 0.8	8.9 ± 1.9$^{\#\#}$	8.8 ± 1.4$^{\#}$	8.8 ± 3.1$^{\#}$	6.0 ± 3.0

TRAP, tartrate-resistant acid phosphatase; Control, glycerol application without ligation; Ligature, glycerol application with ligation; TF1, 1 mg/mL TF application with ligation; TF10, 10 mg/mL TF application with ligation; and TF100, 100 mg/mL TF application with ligation.
$^{\#}P < 0.05$, $^{\#\#}P < 0.001$ was considered significantly different compared with the Control group. $^{*}P < 0.05$, $^{**}P < 0.001$ was considered significantly different compared with the Ligature group.

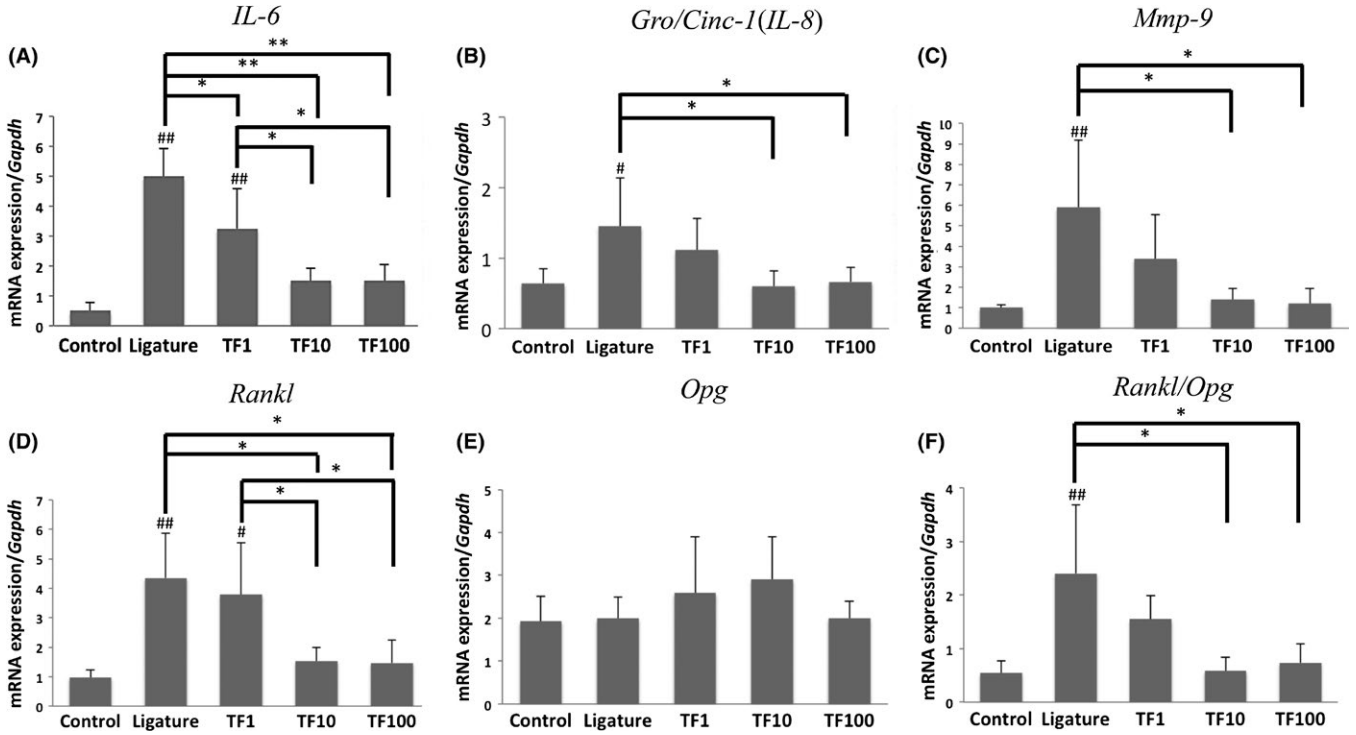

FIGURE 8 Effects of TFs on the mRNA expression of (A) *IL-6*, (B) *Gro/Cinc-1* (*IL-8*), (C) *Mmp-9*, (D) *Rankl*, and (E) *Opg* (F) and the *Rankl/Opg* expression ratio in the gingival tissue of rats, as determined by qPCR. The data were normalized to the housekeeping gene, *Gapdh*. Data are expressed as mean ± SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ compared with the Control group; $^{*}P < 0.05$, $^{**}P < 0.001$ compared with the Ligature group. *IL*, interleukin; *Gro/Cinc-1*, rat equivalent of IL-8; *Mmp*, matrix metalloproteinase; *Rankl*, receptor activator of nuclear factor-kappa B ligand; *Opg*, osteoprotegerin. Control, glycerol application without ligation; Ligature, glycerol application with ligation; TF1, 1 mg/mL TF application with ligation; TF10, 10 mg/mL TF application with ligation; and TF100, 100 mg/mL TF application with ligation

To confirm the antiinflammatory effects of TFs in experimental periodontitis, we evaluated the periodontal tissue by H&E and immunohistochemical staining for CD45, which, as a pan-leukocyte marker, is widely used to evaluate the inflammatory response. A previous study showed the presence of many CD45-positive cells in the gingival biopsies obtained from patients with periodontitis.[41] Moreover, we used qPCR to assess changes in the mRNA expression of inflammatory mediators in gingival tissue. Histological observations demonstrated that the TF10 and TF100 groups had decreased inflammatory cell infiltration and a decreased distance of apical migration of the JE compared with the Ligature and TF1 groups. These results were further confirmed by the observation and quantification of CD45-positive cells. Similar results were observed with inflammatory mediators in gingival tissues. In the Ligature group, the mRNA expression of *IL-6*, *Gro/Cinc-1*(*IL-8*), and *Mmp-9* was significantly increased compared with the Control group, but was significantly downregulated in the TF10 and TF100 groups. These results are in agreement with previous studies, which reported that TFs can inhibit *IL-6* expression by downregulating NF-κB activation.[20,21,42] In addition, TFs can attenuate the secretion of *IL-8* in oral epithelial cells after stimulation with LPS by inhibiting activation of the IkappaB kinase and activator protein-1 pathways.[25,43] Recently, Ben Lagha and Grenier[27] reported that TFs can inhibit IL-6, IL-8, and MMP-9 secretion from *P. gingivalis*-stimulated macrophages, mostly likely by blocking activation of the NF-κB signaling pathway. These data indicate that TFs may play a significant role in antiinflammatory effects.

Next, we investigated the effects of TFs on osteoclast formation and alveolar bone resorption. First, we determined the osteoclast cell number by TRAP staining, as TRAP is an important cytochemical marker of osteoclasts. The Control group exhibited several TRAP-positive osteoclasts on the alveolar bone surface, which corresponded to normal bone metabolism. On the other hand, the Ligature group had many TRAP-positive osteoclasts on both segments, especially on the Line X segments. This may have been due to its proximity to the ligated suture, which induced inflammatory cell infiltration. Therefore, we separated the Line X and Line Y segments to evaluate the effect of TFs in different regions. As expected, the numbers of TRAP-positive cells in the TF10 and TF100 groups were significantly lower than those in the Ligature group on the Line X segment. Interestingly, on the Line Y segment, all other groups with ligature placement, with the exception of the TF100 group, had significantly increased numbers of TRAP-positive cells compared with the Control group. This may have been due to the concentration gradient of TFs, as high concentrations of TFs can taper down to more distant sites on the Line Y segment. Consistent with the histological findings by TRAP staining, micro-CT analysis showed that the TF10 and TF100 groups had less alveolar bone resorption and a higher BV/TV ratio than the Ligature and TF1 groups.

To understand the mechanisms underlying osteoclast differentiation and formation, osteoclastogenesis-related genes in gingival tissues were evaluated by qPCR. The critical factor in bone resorption is the *Rankl/Opg* ratio.[44] In periodontitis, RANKL is upregulated, whereas OPG is downregulated compared to healthy periodontal tissue, resulting in an increased RANKL/OPG ratio.[45] In this study, the TF10 and TF100 groups had less expression of *Rankl* than the Ligature and TF1 groups. On the other hand, *Opg* expression tended to increase in all TF groups, but the difference was not significant. The TF10 and TF100 groups had attenuation of the *Rankl/Opg* ratio compared with the Ligature group. Therefore, inhibition of *Rankl* expression by TFs may be an important mechanism underlying the reduction in osteoclast differentiation, formation, and bone loss in experimental periodontitis.

Taken together, our results suggest that TFs may play important roles in inhibiting alveolar bone resorption, possibly by suppressing inflammatory mediators that cause osteoclastogenesis.[46] Expression of the inflammatory mediators and the *Rankl/Opg* expression ratio, as determined by qPCR, was consistent with the number of TRAP-positive cells on the Line X segment. TFs have recently received much attention regarding their ability to inhibit osteoclast cell formation and differentiation. TF-3 can inhibit the expression of osteoclast-related genes including TRAP by suppressing the ERK pathway.[47] Nishikawa et al[48] showed that TF-3, which selectively reduces Dnmt3 methyltransferase activity stimulated by RANKL, can reduce osteoclast differentiation. These studies reinforce the theory that TFs can directly act on osteoclasts. Additional studies are required to better understand the effects of TFs in periodontal disease, and to determine whether TFs can act directly on osteoclasts in ligature-induced experimental periodontitis.

In conclusion, the results of this study demonstrate that the topical application of TFs significantly reduces periodontal tissue inflammation and alveolar bone loss, suggesting that TFs have therapeutic effects in periodontal disease.

ACKNOWLEDGEMENTS

We thank Yaizu Suisankagaku Industry, Shizuoka, Japan, for kindly providing the TFs. We are also grateful to Dr. Shuichi Hashimoto (The Nippon Dental University) for assisting with data acquisition and providing advice. This study was conducted using the facilities and financial support of The Nippon Dental University School of Life Dentistry at Tokyo.

CONFLICT OF INTEREST

The authors have no conflict of interests to declare.

ORCID

Ya-Hsin Wu http://orcid.org/0000-0002-4451-7736
Ryutaro Kuraji http://orcid.org/0000-0002-7900-3604

REFERENCES

1. Williams RC. Periodontal disease. *N Engl J Med*. 1990;322:373-382.
2. Silva N, Abusleme L, Bravo D, et al. Host response mechanisms in periodontal diseases. *J Appl Oral Sci*. 2015;23:329-355.
3. Dongari-Bagtzoglou AI, Ebersole JL. Increased presence of interleukin-6 (IL-6) and IL-8 secreting fibroblast subpopulations in adult periodontitis. *J Periodontol*. 1998;69:899-910.
4. de Oliveira PA, de Pizzol-Junior JP, Longhini R, Sasso-Cerri E, Cerri PS. Cimetidine reduces interleukin-6, matrix metalloproteinases-1 and -9 immunoexpression in the gingival mucosa of rat molars with induced periodontal disease. *J Periodontol*. 2017;88:100-111.
5. Chakrabarti S, Patel KD. Regulation of matrix metalloproteinase-9 release from IL-8-stimulated human neutrophils. *J Leukoc Biol*. 2005;78:279-288.
6. Franco C, Patricia HR, Timo S, Claudia B, Marcela H. Matrix metalloproteinases as regulators of periodontal inflammation. *Int J Mol Sci*. 2017;18:440.
7. Liu W, Zhang X. Receptor activator of nuclear factor-κB ligand (RANKL)/RANK/osteoprotegerin system in bone and other tissues (review). *Mol Med Rep*. 2015;11:3212-3218.
8. Mori T, Miyamoto T, Yoshida H, et al. IL-1beta and TNFalpha-initiated IL-6-STAT3 pathway is critical in mediating inflammatory cytokines and RANKL expression in inflammatory arthritis. *Int Immunol*. 2011;23:701-712.
9. Nakashima T, Kobayashi Y, Yamasaki S, et al. Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-kappaB ligand: modulation of the expression by osteotropic factors and cytokines. *Biochem Biophys Res Commun*. 2000;275:768-775.
10. Bartold PM, Cantley MD, Haynes DR. Mechanisms and control of pathologic bone loss in periodontitis. *Periodontol 2000*. 2010;53:55-69.
11. Loesche WJ, Grossman NS. Periodontal disease as a specific, albeit chronic, infection: diagnosis and treatment. *Clin Microbiol Rev*. 2001;14:727-752, table of contents.
12. Souza JA, Rossa C Jr, Garlet GP, Nogueira AV, Cirelli JA. Modulation of host cell signaling pathways as a therapeutic approach in periodontal disease. *J Appl Oral Sci*. 2012;20:128-138.
13. Guimaraes MR, Coimbra LS, de Aquino SG, Spolidorio LC, Kirkwood KL, Rossa C Jr. Potent anti-inflammatory effects of systemically administered curcumin modulate periodontal disease in vivo. *J Periodontal Res*. 2011;46:269-279.
14. Palaska I, Papathanasiou E, Theoharides TC. Use of polyphenols in periodontal inflammation. *Eur J Pharmacol*. 2013;720:77-83.
15. Varoni EM, Lodi G, Sardella A, Carrassi A, Iriti M. Plant polyphenols and oral health: old phytochemicals for new fields. *Curr Med Chem*. 2012;19:1706-1720.
16. Kaye EK. Nutrition, dietary guidelines and optimal periodontal health. *Periodontol 2000*. 2012;58:93-111.
17. Yang CS, Landau JM. Effects of tea consumption on nutrition and health. *J Nutr*. 2000;130:2409-2412.
18. Sang S, Lambert JD, Ho CT, Yang CS. The chemistry and biotransformation of tea constituents. *Pharmacol Res*. 2011;64:87-99.
19. Leung LK, Su Y, Chen R, Zhang Z, Huang Y, Chen ZY. Theaflavins in black tea and catechins in green tea are equally effective antioxidants. *J Nutr*. 2001;131:2248-2251.
20. Kim S, Joo YE. Theaflavin inhibits LPS-induced IL-6, MCP-1, and ICAM-1 expression in bone marrow-derived macrophages through the blockade of NF-kappaB and MAPK signaling pathways. *Chonnam Med J*. 2011;47:104-110.
21. Wu Y, Jin F, Wang Y, et al. In vitro and in vivo anti-inflammatory effects of theaflavin-3,3′-digallate on lipopolysaccharide-induced inflammation. *Eur J Pharmacol*. 2017;794:52-60.

22. Lahiry L, Saha B, Chakraborty J, et al. Theaflavins target Fas/caspase-8 and Akt/pBad pathways to induce apoptosis in p53-mutated human breast cancer cells. *Carcinogenesis*. 2010;31:259-268.
23. Pan MH, Lin-Shiau SY, Ho CT, Lin JH, Lin JK. Suppression of lipopolysaccharide-induced nuclear factor-kappaB activity by theaflavin-3,3′-digallate from black tea and other polyphenols through down-regulation of IkappaB kinase activity in macrophages. *Biochem Pharmacol*. 2000;59:357-367.
24. Oka Y, Iwai S, Amano H, et al. Tea polyphenols inhibit rat osteoclast formation and differentiation. *J Pharmacol Sci*. 2012;118:55-64.
25. Lombardo Bedran TB, Morin MP, Palomari Spolidorio D, Grenier D. Black tea extract and its theaflavin derivatives inhibit the growth of periodontopathogens and modulate interleukin-8 and beta-defensin secretion in oral epithelial cells. *PLoS ONE*. 2015;10:e0143158.
26. Hosokawa Y, Hosokawa I, Ozaki K, Nakanishi T, Nakae H, Matsuo T. Tea polyphenols inhibit IL-6 production in tumor necrosis factor superfamily 14-stimulated human gingival fibroblasts. *Mol Nutr Food Res*. 2010;54(Suppl 2):S151-S158.
27. Ben Lagha A, Grenier D. Black tea theaflavins attenuate *Porphyromonas gingivalis* virulence properties, modulate gingival keratinocyte tight junction integrity and exert anti-inflammatory activity. *J Periodontal Res*. 2017;52:458-470.
28. Yoshinaga Y, Ukai T, Nakatsu S, et al. Green tea extract inhibits the onset of periodontal destruction in rat experimental periodontitis. *J Periodontal Res*. 2014;49:652-659.
29. Morin MP, Grenier D. Regulation of matrix metalloproteinase secretion by green tea catechins in a three-dimensional co-culture model of macrophages and gingival fibroblasts. *Arch Oral Biol*. 2017;75:89-99.
30. Rattanasuwan K, Rassameemasmaung S, Sangalungkarn V, Komoltri C. Clinical effect of locally delivered gel containing green tea extract as an adjunct to non-surgical periodontal treatment. *Odontology*. 2016;104:89-97.
31. Kuraji R, Fujita M, Ito H, Hashimoto S, Numabe Y. Effects of experimental periodontitis on the metabolic system in rats with diet-induced obesity (DIO): an analysis of serum biochemical parameters. *Odontology*. 2018;106:162-170.
32. Kuraji R, Ito H, Fujita M, Ishiguro H, Hashimoto S, Numabe Y. *Porphyromonas gingivalis* induced periodontitis exacerbates progression of non-alcoholic steatohepatitis in rats. *Clin Exp Dent Res*. 2016;2:216-225.
33. Sanbe T, Tomofuji T, Ekuni D, Azuma T, Tamaki N, Yamamoto T. Oral administration of vitamin C prevents alveolar bone resorption induced by high dietary cholesterol in rats. *J Periodontol*. 2007;78:2165-2170.
34. Himi T, Yoshioka I, Kataura A. Production and gene expression of IL-8-like cytokine GRO/CINC-1 in rat nasal mucosa. *Acta Otolaryngol*. 1997;117:123-127.
35. Kuhr A, Popa-Wagner A, Schmoll H, Schwahn C, Kocher T. Observations on experimental marginal periodontitis in rats. *J Periodontal Res*. 2004;39:101-106.
36. de Molon RS, de Avila ED, Cirelli JA. Host responses induced by different animal models of periodontal disease: a literature review. *J Investig Clin Dent*. 2013;4:211-218.
37. Liu L, Li C, Cai X, Xiang J, Cao Z, Dong W. The temporal expression and localization of extracellular matrix metalloproteinase inducer (EMMPRIN) during the development of periodontitis in an animal model. *J Periodontal Res*. 2010;45:541-549.
38. Wang W, Sun Y, Liu J, et al. Protective effect of theaflavins on cadmium-induced testicular toxicity in male rats. *Food Chem Toxicol*. 2012;50:3243-3250.
39. Mulder TP, van Platerink CJ, Wijnand Schuyl PJ, van Amelsvoort JM. Analysis of theaflavins in biological fluids using liquid chromatography-electrospray mass spectrometry. *J Chromatogr B Biomed Sci Appl*. 2001;760:271-279.
40. Pereira-Caro G, Moreno-Rojas JM, Brindani N, et al. Bioavailability of black tea theaflavins: absorption, metabolism, and colonic catabolism. *J Agric Food Chem*. 2017;65:5365-5374.
41. Bage T, Kats A, Lopez BS, et al. Expression of prostaglandin E synthases in periodontitis immunolocalization and cellular regulation. *Am J Pathol*. 2011;178:1676-1688.
42. Song YA, Park YL, Yoon SH, et al. Black tea polyphenol theaflavin suppresses LPS-induced ICAM-1 and VCAM-1 expression via blockage of NF-kappaB and JNK activation in intestinal epithelial cells. *Inflamm Res*. 2011;60:493-500.
43. Aneja R, Odoms K, Denenberg AG, Wong HR. Theaflavin, a black tea extract, is a novel anti-inflammatory compound. *Crit Care Med*. 2004;32:2097-2103.
44. Cochran DL. Inflammation and bone loss in periodontal disease. *J Periodontol*. 2008;79:1569-1576.
45. Belibasakis GN, Bostanci N. The RANKL-OPG system in clinical periodontology. *J Clin Periodontol*. 2012;39:239-248.
46. Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. *Nat Rev Immunol*. 2007;7:292-304.
47. Hu X, Ping Z, Gan M, et al. Theaflavin-3,3′-digallate represses osteoclastogenesis and prevents wear debris-induced osteolysis via suppression of ERK pathway. *Acta Biomater*. 2017;48:479-488.
48. Nishikawa K, Iwamoto Y, Kobayashi Y, et al. DNA methyltransferase 3a regulates osteoclast differentiation by coupling to an S-adenosylmethionine-producing metabolic pathway. *Nat Med*. 2015;21:281-287.

How to cite this article: Wu Y-H, Kuraji R, Taya Y, Ito H, Numabe Y. Effects of theaflavins on tissue inflammation and bone resorption on experimental periodontitis in rats. *J Periodont Res*. 2018;53:1009–1019. https://doi.org/10.1111/jre.12600