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The effect of orally administered epigallocatechin-3-gallate on ligature-induced periodontitis in rats

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Background and Objective: Epigallocatechin-3-gallate (EGCG) is known for its beneficial properties, including anti-inflammatory and anti-oxidative activities. Recently, reports have suggested that EGCG plays a pivotal role in regulating cytokine expression and osteoclastic activity. In the present study, we investigated whether orally administered EGCG has a therapeutic effect on ligature-induced periodontitis.

Materials and Methods: Forty-eight Sprague–Dawley rats were treated with EGCG or phosphate-buffered saline. Periodontitis was induced by tying a ligature for 7 d. After removing ligation, EGCG (200 mg/kg) or phosphate-buffered saline was administered via oral gavage on a daily basis. Rats were killed after 1, 2 and 4 wk of administration. Histologic and histomorphometric analyses, tartrate resistant acid phosphatase staining and immunohistochemistry were carried out.

Results: In the control group, bone loss did not recover even after the causative factor of periodontitis was eliminated. On the other hand, distance from cemento-enamel junction to alveolar bone crest, long junctional epithelium and collagen destruction were reduced in the EGCG group. Decreased interleukin (IL)-6 expression was shown from the early stage of EGCG administration, followed by reduced tumor necrosis factor (TNF) expression at week 4 EGCG group. The CT area showed a higher decrease of IL-6 expression between the control and EGCG group than alveolar bone area. Downregulation of TNF and IL-6 expression led to a decrease in osteoclast number and activity, which resulted in reduced bone loss.

Conclusions: Systemic administration of EGCG could have a therapeutic effect on damaged periodontal tissue. Inhibited cytokine expression, including TNF and IL-6 is responsible for the reduction in osteoclast formation, osteoclastic activity and collagen destruction.

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Periodontitis is one of the most prevalent chronic inflammatory diseases and is initiated by pathogenic biofilm accumulation (1). When periodontal pathogens such as *Porphyromonas gingivalis* invade into the periodontium, alterations in the number and composition of the oral commensal microbiota are instigated, causing dysbiosis

and activation of host response (2,3). Although the initial response involves periodontal pathogens, a series of host responses, including inflammatory cell infiltration, cytokine secretion and activation of osteoclasts, are chiefly responsible for the progression of periodontal disease (4–6). These eventually lead to the breakdown of tooth-supporting structures, including periodontal connective tissue (CT) and alveolar bone (AB).

To promote periodontal treatment and regeneration, a number of surgical and nonsurgical periodontal therapies such as bone grafting, growth factor application and laser therapies have been investigated (7,8). Although these methods of treatment are effective, less aggressive and biocompatible pharmacological treatments are of interest and remain to be developed to use as independent or supplementary periodontal treatments. Antibiotics (9), vitamins (10) and other natural products, including cocoa (11), chitosan (12) and green tea extract (13) are a few examples of pharmacological treatments for periodontal disease.

Epigallocatechin-3-gallate (EGCG), the major catechin substance from green tea, has many pharmacological effects, which have been reported to promote overall health. Green tea has been well documented for its antioxidant, anticarcinogenic, antimicrobial and anti-inflammatory properties (14). Owing to its readily accessible and biocompatible properties, there is an increased interest in EGCG as a pharmaceutical agent in oral medicine. Gillespie *et al.* (15) reported that systemic EGCG consumption could be useful in delaying Sjögren's syndrome by reducing autoimmune-induced lymphocyte infiltration and inhibiting cell proliferation. Furthermore, the preventive effect of green tea catechin-containing dentifrices was evaluated during topical application (16).

Recently, the underlying mechanisms of the preventive effects of EGCG on tissue destruction have been explained. Some studies found that EGCG suppressed cytokine productions such as interleukin (IL)-1β, tumor necrosis factor (TNF) and IL-6, which in turn accelerated the progression of inflammation and bone resorption (17–19). In addition, several *in vitro* studies have confirmed the effects of EGCG on osteoclast formation. Oka *et al.* (20) examined whether EGCG has an effect on osteoclast precursor cell and osteoclast, and concluded that EGCG has an inhibitory effect on formation and differentiation of osteoclasts via matrix metalloproteinase (MMP) suppression. As osteoblasts are known to be involved in the differentiation of osteoclasts through the production of receptor activator of nuclear factor-kappa B ligand (RANKL), Kamon *et al.* (21) have also reported that EGCG reduced osteoclast formation by inhibiting osteoblast differentiation. However, many of these studies are limited to *in vitro* techniques in which few studies reported the effect of pure EGCG on an experimental periodontitis model.

As EGCG has been proven to have much pharmacological potential, we hypothesized that the administration of EGCG will have a positive effect on improving pathological periodontal tissue. The aim of the present study was to investigate whether orally administered EGCG has a therapeutic effect on ligature-induced periodontitis.

Material and methods

Animals

Forty-eight male Sprague–Dawley rats (150–180 g) were fed food and water *ad libitum*. The animals were maintained in a temperature-controlled room (22°C) on a 12 h light–dark cycle. The animal experiment was conducted in accordance with guidelines approved by the Animal Ethics Committee of the Yonsei University College of Dentistry, Seoul, Korea (2010-0388).

Materials

EGCG (99% purity, Chengdu Biopurify Phytochemicals Ltd., Sichuan, China) was prepared and diluted in phosphate-buffered saline (PBS) (22). EGCG, 200 mg/kg of body weight of the animal, was administered and the dose was chosen based on previous studies in published literature and our pilot study (23,24). The EGCG solution was diluted just before administration to avoid degradation of EGCG (25).

Study design

Rats were randomly divided into two groups: (i) control group ($n = 24$; animals were fed PBS vehicle after inducing experimental periodontitis), and (ii) EGCG group ($n = 24$; animals were fed EGCG after inducing experimental periodontitis). Rats were killed 1, 2 and 4 wk after EGCG or PBS administration.

Experimental methods

Ligature-induced periodontitis— Rats were intraperitoneally anesthetized with a 2 : 3 mixture of rompun and zoletil to induce experimental periodontitis. A sterilized nylon thread ligature was placed around the cervix of the mandibular left or right first molar and knotted mesially as previously described (26). The ligatures were kept in subgingival positions for 7 d to cause accumulation of dental plaque (27). The ligatures were removed before administration of EGCG.

Administration of epigallocatechin-3-gallate— EGCG diluted in PBS was administered to the EGCG group and a PBS vehicle was given to rats in the control group. All administration was conducted via oral gavage on a daily basis. The total volume of daily gavage was 2.0 mL. Rats were killed by CO_2 inhalation 1, 2 and 4 wk after administration (Fig. 1). Body weights were measured daily throughout the experiment to monitor any unexpected symptoms.

Analysis

Histological and histomorphometric analysis— The animals were killed after the experimental period. Tissue samples containing mandibular molars, AB and surrounding soft tissues were

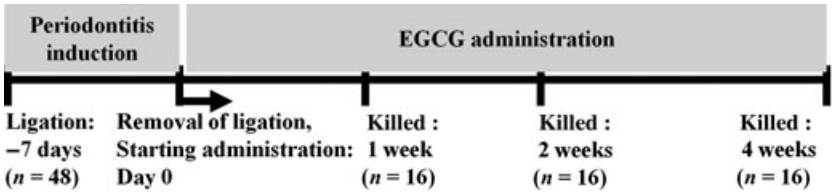

Fig. 1. Study design. Forty-eight rats were divided into two groups. After inducing periodontitis, eight rats from each group were killed at 1, 2, and 4 wk within the period of administration. EGCG, epigallocatechin-3-gallate.

dissected from the left and right side of the mandible and fixed in 10% buffered formaldehyde (pH 7.4) for 1 d. Samples were then decalcified with 10% tetrasodium-EDTA aqueous solution (pH 7.4) for 2 mo at room temperature. The tissue blocks were embedded in paraffin, and serial mesiodistal sections (5 μm) were stained with hematoxylin and eosin.

Interdental areas between the first and second molars of the mandible were examined at a magnification of 40× using light microscopy (Olympus multiview microscope BH2, Tokyo, Japan) and PC-based image analysis system (Image-Pro Plus, Media Cybernetic, Silver Spring, MD, USA). Measurements included the degree of apical migration of the junctional epithelium (long junctional epithelium) and the AB level for evaluation. The distance between the cemento-enamel junction and the most apical portion of the junctional epithelium (long junctional epithelium) and that between the cemento-enamel junction and the AB crest were measured (28) (Fig. 2). The results were presented in millimeters.

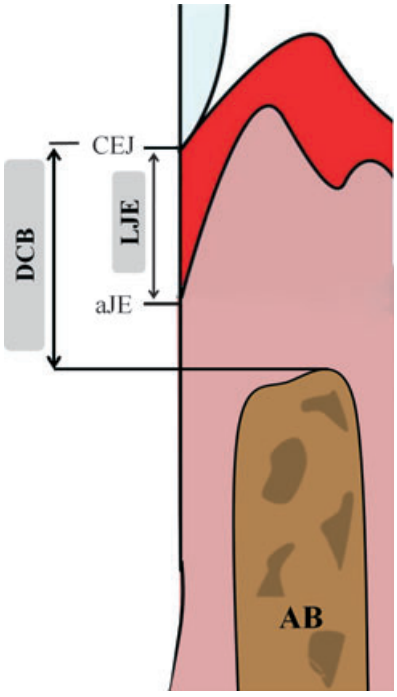

Fig. 2. Schematic drawing of the histometric analysis. AB, alveolar bone; aJE, most apically positioned junctional epithelium; CEJ, cemento-enamel junction; DCB, distance from CEJ to alveolar bone crest; LJE, long junctional epithelium.

Immunohistochemistry— Immunohistochemical staining for TNF and IL-6 was conducted on both the experimental and control groups. Deparaffinized tissue sections were incubated with 2% H_2O_2 for 30 min and then treated with 0.02 mg/mL of proteinase K (Sigma, St Louis, MO, USA) for 10 min at 25°C. They were immersed in primary anti-TNF antibody (ab1793; Abcam, Cambridge, UK) and IL-6 (SC-1265, Santa Cruz Biotechnology, CA, USA) antibody for 30 min at room temperature. After washing in PBS, a biotin-conjugated goat secondary antibody (sc-2040; Santa Cruz Biotechnology) was applied, followed by incubation at room temperature for 60 min. The immunohistochemical reactions were developed in 3,3′-diaminobenzidine tetrahydrochloride. Slides were counterstained with hematoxylin. Captured images (magnification 400×) on CT area and AB area were evaluated respectively. The AB area includes the periodontal ligament space and AB crest. Cells with strong or moderate brown staining were counted as positive (29).

Osteoclastic activity— Tartrate resistant acid phosphatase (TRAP) staining was used to identify osteoclastic activity. Staining was followed by recommended protocol using an acid phosphatase kit (Sigma Chemical Co., St Louis, MO, USA). After rehydration, tissue sections were permeabilized using 0.1% TritonX 100 for 15 min, followed by a PBS wash and incubation with TRAP reagents for up to 1 h. Sections were washed and counterstained with hematoxylin. Osteoclasts are defined as TRAP-positive multinucleated cells adjacent to bone surfaces. TRAP-positive surface and the number of osteoclasts were measured for quantitative analysis (30,31). Measurements were performed from the AB crest to below the crest region and the area of interest was restricted within 0.5 mm × 2.5 mm.

Statistical analysis

Results are presented as the means ± standard error (SE). Student's *t*-tests and Mann–Whitney *U* test were performed to compare two mean values. *P* values of < 0.05 were accepted as statistically significant.

Results

Clinical findings

Animals showed no signs of systemic illness and gained weight normally during the experiment period. The regions with tied ligatures showed swelling and bleeding on probing.

Histological and histomorphometric analysis

We analyzed EGCG-mediated histological changes in ligature-induced periodontitis. In the control group, the bone resorptive process and collagen denaturation showed active progress until 2 wk after removing the ligature. At week 1, the surface of the root displayed an evident resorptive pit and irregular cementum. Vasodilatation was observed, but evident inflammatory infiltrates were not found in any of the control groups (Fig. 3A–C). The 2 wk control group had the longest

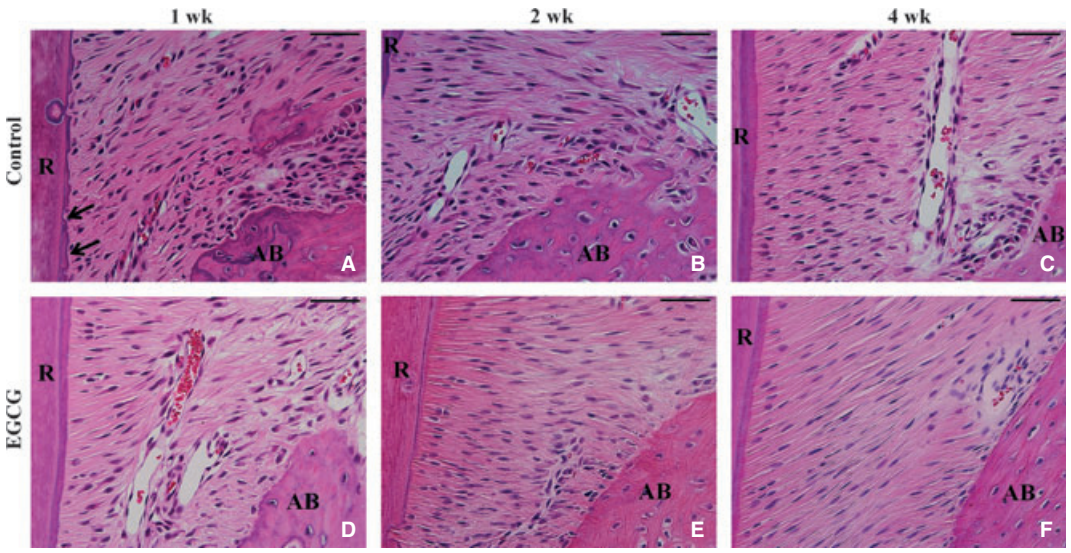

Fig. 3. Histological analysis of ligatured interdental area in control and EGCG group. Control at: (A) week 1; (B) week 2; and (C) week 4. EGCG at: (D) week 1; (E) week 2; and (F) week 4. Arrows indicate irregular cementum. Scale bars represent 50 μm. AB, alveolar bone; EGCG, epigallocatechin-3-gallate; R, distal root of first molar.

distance from the cemento-enamel junction to the AB crest and long junctional epithelium, whereas the 4 wk control group decreased in its length (Fig. 4A,B). That is, the lowest bone level was observed at 2 wk after removing ligation among the three time-specific control groups. In the EGCG group, the 1 wk subgroup showed lower bone loss than the 1 wk control group, but it displayed similar collagen destruction with the 4 wk control group (Fig. 3D). Bone loss significantly decreased 2 wk after EGCG administration ($p = 0.0001$). The arrangement of collagen was regular and collagen fibers were inserted vertically into the cementum with the AB showing Sharpey's fibers (Fig. 3E). Irregular collagen fibers, inflammatory cell infiltration and resorptive bone surfaces were not found at week 4 (Fig. 3F). Apical migration of junctional epithelium in all of the control groups was greater than those in EGCG groups where there was a significant difference at weeks 1 ($p = 0.023$) and 2 ($p = 0.005$).

Immunohistochemistry

Measurements were divided into two parts to compare the expression of cytokines (TNF and IL-6) in the CT and AB areas. The TNF immunohistochemistry resulted in no significant differences in most of the control and EGCG groups (AB area and CT area). However, week 4 staining showed that there was a significant difference (CT area, $p = 0.004$; AB area, $p = 0.009$) between the control group and the EGCG group as there was an increase

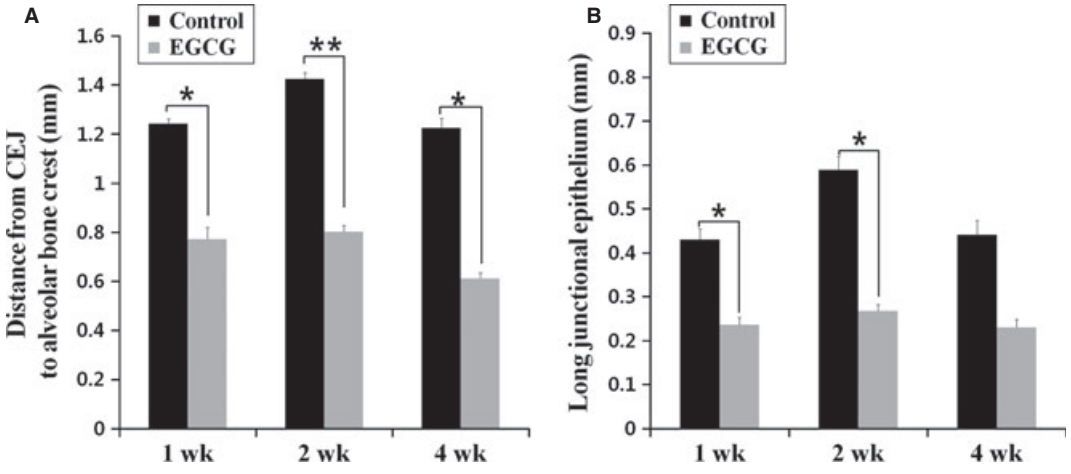

Fig. 4. Histomorphometry analysis of control and EGCG group. Mean value (A) of distance from CEJ to alveolar bone crest, and (B) long junctional epithelium. *$p < 0.05$ compared to control group, **$p < 0.001$ compared to control group. CEJ, cement–enamel junction; EGCG, epigallocatechin-3-gallate.

in the control group from weeks 2 to 4, but a decrease in the EGCG group from weeks 2 to 4 (Fig. 5A,B). This demonstrates that the administration of EGCG for 4 wk may significantly decrease TNF expression compared to the control group. The EGCG group showed low expression of IL-6 in the CT area at weeks 1, 2 and 4. Significant differences were found between the control and EGCG group at all durations (week 1, $p = 0.001$; weeks 2 and 4, $p = 0.002$) (Fig. 5C). In the AB area, the control group showed a peak expression at week 2. A decreasing pattern could be observed in IL-6 expression of the EGCG group throughout the weeks 1, 2 and 4. There was a significant difference between the control and EGCG group at weeks 2 and 4 (week 2, $p = 0.001$; week 4, $p = 0.028$) (Fig. 5D). The CT area showed a three-fold higher decrease of IL-6 expression between the control group and EGCG group than the AB area.

Osteoclastic activity

TRAP staining was performed to identify osteoclastic activity and was indicated with a purple stain. Osteoclasts were mainly positioned on the mesial surface of the AB. In the control group, osteoclastic multinucleated cells were within the ruffled border that showed strong TRAP-positive reactions (Fig. 6A–C). Following EGCG administration, TRAP-negative cells were observed in addition to a decrease in osteoclast number (Fig. 6D–F). Osteoclast number and osteoclastic surface increased and exhibited a peak level at week 2 in the control group, even though those at week 4 lessened slightly. On the contrary, the EGCG group generally showed a time-dependent decrease and a lower TRAP-positive activity compared to the control group (Fig. 7A,B). There was a statistical significance in the number of osteoclasts at weeks 2 ($p = 0.001$) and 4 ($p = 0.019$), albeit the TRAP-positive surface was only significantly different at week 2 ($p = 0.029$).

Discussion

In the present study, we investigated the therapeutic effect of EGCG on periodontal disease when taken systemically on a daily basis. To identify the effect of EGCG on periodontal disease, we used the rat ligature-induced periodontitis model. The advantage of the *in vivo* periodontitis model using ligation is that it imitates causative factors in the oral environment that promote the accumulation of plaque in human periodontitis (32). It has been reported that the peak duration of bone and attachment loss showed within a 7 d period, and osteoclasts and lymphocytes were mainly found within a 7–11 d period (27,33). Therefore, a nylon thread ligature was tied for 7 d to induce periodontitis and ligation was removed before

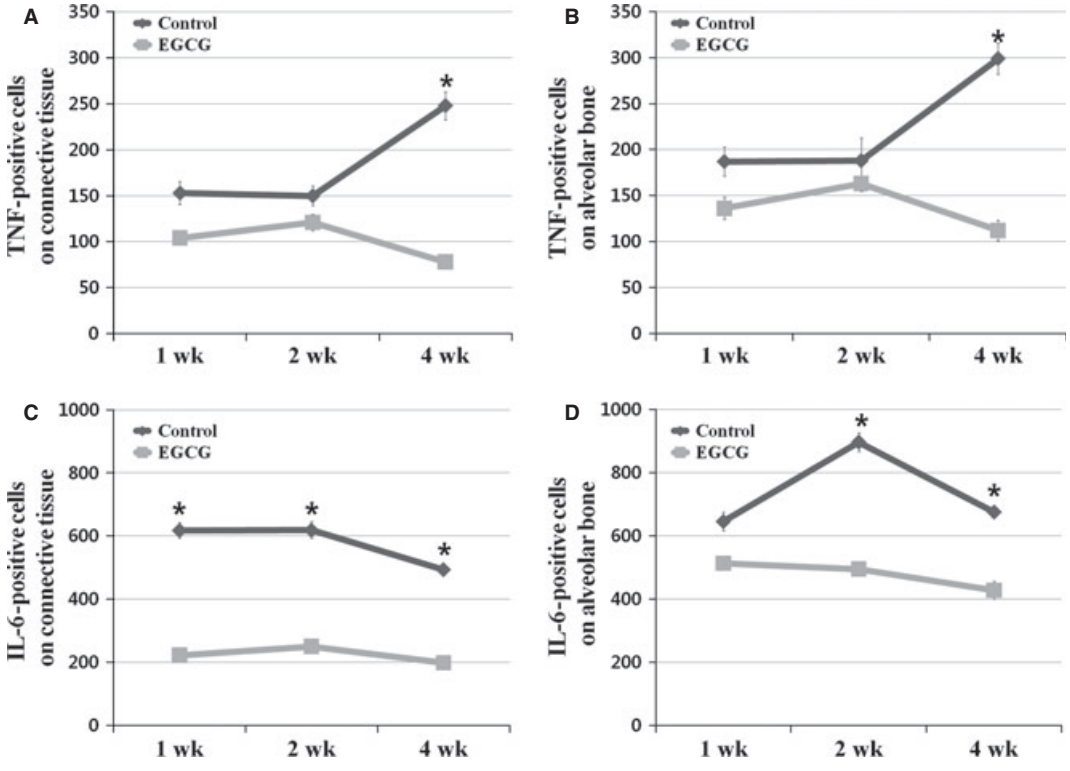

Fig. 5. Quantitative analysis of TNF and IL-6 immunostaining. Number of: (A) TNF-positive cells in connective tissue area; (B) TNF-positive cells in alveolar bone area; (C) IL-6-positive cells in connective tissue area; and (D) IL-6-positive cells in alveolar bone area. *$p < 0.05$ compared to control group. EGCG, epigallocatechin-3-gallate; IL, interleukin; TNF, tumor necrosis factor.

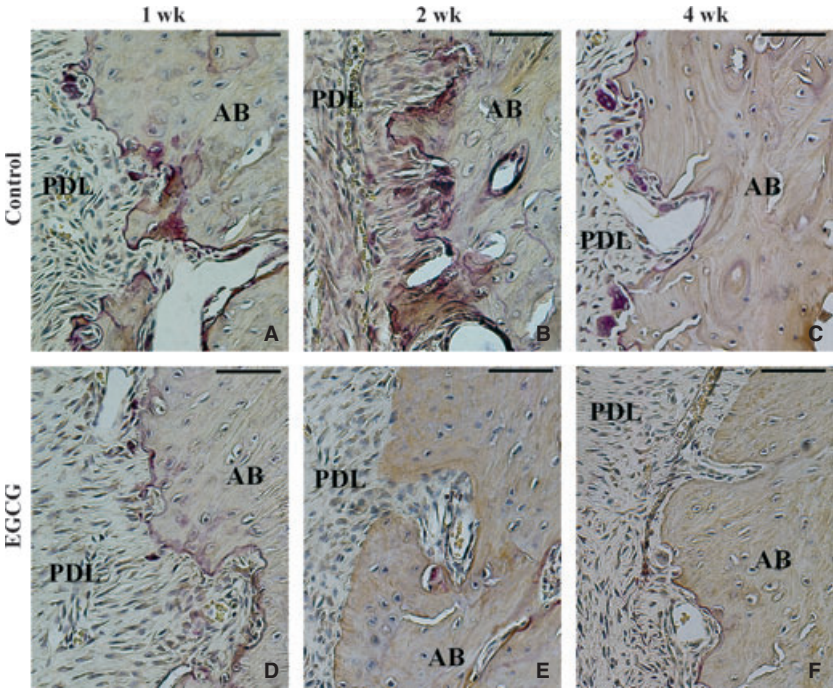

Fig. 6. Histological photographs of tartrate resistant acid phosphatase-stained sections. Control at: (A) week 1; (B) week 2; and (C) week 4. EGCG at: (D) week 1; (E) week 2; and (F) week 4. Tartrate resistant acid phosphatase-positive osteoclasts and surface stained with violet color. AB, alveolar bone; EGCG, epigallocatechin-3-gallate; PDL, periodontal ligament space. Scale bars represent 200 μm.

beginning EGCG feeding to confirm the effect of EGCG administration. Previous study by Tokunaga *et al*. (7) reported that they induced periodontitis using a 20 d ligation and height of AB did not recover in 35–70 d after removing the ligation. Our data showed that levels of bone and attachment loss in controls did not recover during the experimental period, reaching a maximum between weeks 1 and 2. This may be due to the remaining irritants on periodontal lesions until the second week, which further aggravated bone and attachment loss even though the causative factor was eliminated. In addition, the effects of EGCG on reducing bone and attachment loss appeared early in week 1 of the EGCG group. However, histological results showed that periodontal tissue recovered displaying a regular arrangement of collagen fibers and a non-ruffled border in the 2 and 4 wk of the EGCG group. This indicates that administration of EGCG for 1 wk may not be enough to repair damaged tissue.

TNF is a proinflammatory cytokine that stimulates MMP expression resulting in collagen denaturation as well as an increase in osteoclastic activity (15,34). Along with TNF, IL-6 is regarded as a key cytokine involved in regulating bone metabolism. IL-6 could be an autocrine and/or paracrine factor in bone resorption, which accelerates osteoclast formation and activation (35). Moreover, the IL-6 derived from fibroblasts is crucial for the augmentation of MMP-1 expression, which causes periodontal tissue destruction (36). Fibroblasts are present in both periodontal ligament space and gingival CT, but IL-6 release is possibly higher in inflamed CT due to an increased composition of fibroblasts. Furthermore, an *in vitro* study demonstrated that EGCG attenuated IL-6 production when human gingival fibroblasts were stimulated with TNF superfamily 14 (19). Therefore, we postulated that the inhibitory effect of IL-6 expression by EGCG treatment would be greater in the CT than in the AB area. To confirm our hypothesis, the evaluation of

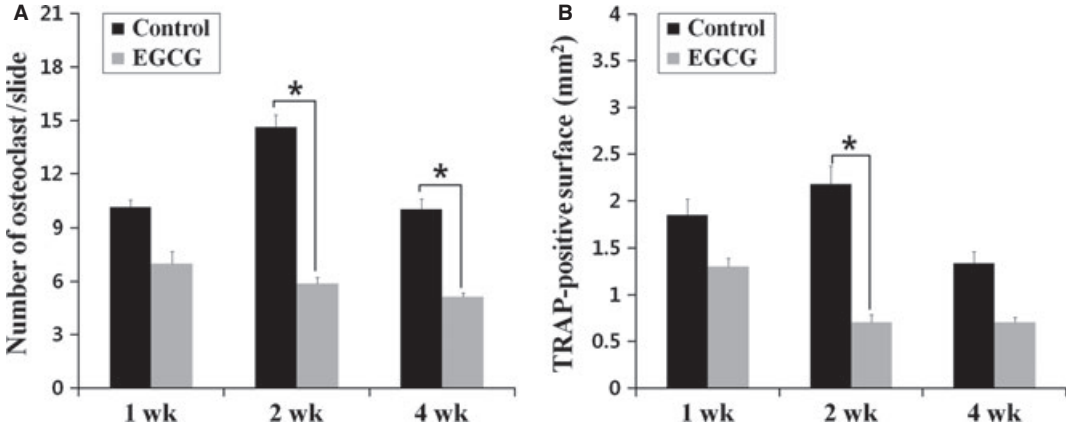

Fig. 7. Quantitative analysis of TRAP staining. Mean value of (A) osteoclast number, and (B) TRAP-positive area. *$p < 0.05$ compared to control group. EGCG, epigallocatechin-3-gallate; TRAP, tartrate resistant acid phosphatase.

TNF and IL-6 expression was divided into two parts: CT and AB areas. TNF expression appeared in similar patterns in both the CT and the AB area, and there was an increase at week 4 of the control group, which was significantly different from the EGCG group. Kon *et al*. (37) analyzed the pattern of TNF expression during the bone repair process. They reported that increased TNF expression was observed at a very early phase within 24 h and a late phase between days 21 and 28 after damage to the bone. Our data with respect to TNF expression in the control group also showed similar patterns of results as the previous study, which suggests that EGCG may prevent an increase in TNF expression during the late healing process. Although the change in TNF expression was seen at week 4, the expression of IL-6 significantly lowered in the 1 or 2 wk EGCG group compared to the control group. This may suggest that administration of EGCG has a direct inhibiting effect on IL-6 levels. Moreover, downregulated IL-6 levels by EGCG in the CT area showed lower levels with an earlier statistical significance compared to its levels in the AB area. This may indicate that the IL-6 suppressing effect of EGCG is expressed much earlier with greater impact in the CT than the AB area. The reduction in cytokines by EGCG was consistent with findings of previous *in vitro* studies. Ahmed *et al*. (22) reported that EGCG inhibited IL-6 synthesis and *trans*-signaling in human rheumatoid arthritis synovial fibroblasts. Shin *et al*. (38) demonstrated that EGCG significantly suppressed TNF and IL-6 production in human mast cells. They also reported that the maximal inhibition rate of IL-6 was greater than that of TNF.

The actions of EGCG on inhibition of osteoclast formation have been reported in previous *in vitro* studies (39,40), and indeed our findings revealed decreased osteoclast number and bone resorptive activity. Week 2 of the EGCG group displayed a significant decrease in the TRAP-positive area meaning the area was actively eroded. Moreover, results from TRAP staining were consistent with the analysis of IL-6 immunostaining in the AB area. From these results, we could confirm that the lower expression of IL-6 led to decreased osteoclast formation and activity, leading to decreased bone loss. This notion was supported by Nakamura *et al*. (39) reporting that green tea catechin has an inhibitory effect on bone resorption. They suggested that green tea catechin suppressed osteoclastogenesis directly or inhibited osteoclastogenesis was shown through suppression of cytokine production such as IL-1β. Another possible cause of improved bone levels in the EGCG group is that EGCG could have an effect on osteoclast apoptosis. Nakagawa *et al*. (41) identified that EGCG induced osteoclastic cell death using the Fenton reaction. Yun *et al*. (42) illustrated the inhibitory effect of EGCG on osteoclast survival via caspase-mediated apoptosis. Further *in vivo* study is needed to confirm the apoptotic effect of EGCG on osteoclasts.

In the present study, we confirmed the effect of EGCG on periodontitis when administered orally. Therefore, it is important to consider how systemically administered EGCG could have influence on the local periodontal lesion. There have been some studies regarding absorption and tissue distribution of EGCG in various animal models, which are significant factors for the pharmacological efficacy of EGCG at the target tissue. Previous studies demonstrated that orally administered EGCG was readily absorbed in the gut with the peak plasma level shown to be reached in approximately 1.5 h at a concentration ranging from 1.6% to 14% in rodents and 20% in dogs (43–46). Moreover, some studies suggested that EGCG existed as metabolites in blood circulation, and both EGCG and its metabolites may exert their chemopreventative properties (46,47). When a green tea polyphenol drink was given for a long period, it was found that EGCG concentration in plasma increased until week 2 and then decreased (48). Furthermore, EGCG was found to have a tendency to distribute into peripheral compartments (45). Swezey *et al*. (46) confirmed the transformed metabolites of EGCG still remained over a broad range of tissues, including the skin, eye, esophagus and liver. The concentration of EGCG and its metabolites found in targeted tissues achieved similar levels to that of plasma. Suganuma *et al*. (47) also demonstrated that EGCG was distributed to a wide range of organs, and the tissue concentration of EGCG was enhanced when taken twice with a 6 h interval.

EGCG concentration in local medium such as saliva and gingival crevicular fluid might determine the orally administered EGCG effect. Tea catechin levels in human saliva after drinking green tea were evaluated by a previous study where saliva samples were collected over 3 h following green tea intake (49). The study confirmed that 4.8–22 μg/mL of EGCG was detected in saliva, and suggested this level was twice the magnitude of that of the plasma following ingestion. Consequently, this study confirmed that oral administration of green tea is an effective method of EGCG delivery to the periodontal tissues and oral mucosa by increasing its bioavailability and adsorption. Makimura *et al*. (50) demonstrated that collagenase activity in gingival crevicular fluid was inhibited by tea catechin *in vitro*. Moreover, other *in vitro* studies found that EGCG inhibited the growth and adherence of *Porphyromonas gingivalis* on to the buccal epithelial cells (51), and reduced the production of toxic substances by *P. gingivalis* (52). Several ways of helping orally administered EGCG exert its pharmacological properties on periodontal tissue were investigated, and it was recommended to increase the oral retention time of EGCG solution to enhance the effect of its administration. The exact dosage of EGCG and its pharmacokinetics to achieve the optimum preventive or therapeutic effect on the periodontium should be further investigated for clinical application.

Within the limitations of this study, systemic administration of EGCG could have a therapeutic effect on

periodontal disease by reducing osteoclastic activity and collagen destruction. Moreover, downregulation of TNF and IL-6 expression is responsible for the therapeutic effect of EGCG. Although there was a time-dependent therapeutic effect of EGCG, a significant effect was shown after 2 wk of administration.

Acknowledgements

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