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Ameliorative effect of quercetin on the destruction caused by experimental periodontitis in rats

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Background and Objective: The purpose of this study was to evaluate the effect of quercetin, a flavonol that exhibits anti-inflammatory properties, on experimental periodontal destruction in rats.

Material and Methods: Osteoclast formation on maxillary palatal alveolus was induced with daily lipopolysaccharide (LPS) injections (0, 1 or 5 mg/mL) for 3 d. Five days later, the osteoclasts on bony surfaces were counted after histochemical staining for tartrate-resistant acid phosphatase. The effect of intragastric quercetin on the osteoclast formation was evaluated in the following three groups: quercetin (75 mg/kg/d by oral feeding); LPS (5 mg/mL); and quercetin plus LPS. Moreover, the effect of quercetin on the ligature-induced periodontitis around maxillary second and mandibular first molars was further evaluated by microcomputerized tomography (on days 0, 4, 8 and 12) and by histometry (on day 8).

Results: A dose-dependent increase in osteoclasts occurred after LPS injections. However, quercetin (75 mg/kg) reduced the 5 mg/mL LPS-induced osteoclasts. Using microcomputerized tomography, the bone crest levels at ligation sites were found to be significantly more apical than at the control sites on days 8 and 12; however, the apically located bone crests rebounded in rats from the quercetin-plus-ligation group. Histometry demonstrated significantly more coronal alveolar crest bone levels, less inflammatory cell-infiltrated connective tissue areas and less connective tissue attachments in the ligation-plus-quercetin group compared with those in the ligation group.

Conclusion: As the quercetin could reduce the LPS-induced osteoclast formation and the ligature-enhanced periodontal inflammation and bone loss, we suggest that it may have an ameliorative effect on periodontal destruction.

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Periodontal disease is an inflammatory disease, and the bacterial plaque is the primary etiology. However, this inflammatory condition in periodontium can be modified by systemic diseases or medications. This inflammatory reaction may damage surrounding cells and connective tissue structures, including the alveolar bone, causing tooth loss (1,2). Lipopolysaccharide (LPS) is the major component of the outer membrane of gram-negative bacteria and can elicit strong immune responses in animals. In periodontitis, bacteria and their products induce polymorphonuclear leukocyte infiltration, edema and vascular dilatation in inflamed periodontal tissues

(3). Periodontal disease can be induced in rats by dietary manipulation (4), introduction of pathogenic microorganisms (5), placement of a ligature that acts as a site for bacterial colonization (6) or injection of bacterial toxins (7). It has been suggested that LPS can penetrate gingival connective tissue and induce a local inflammatory response that leads to periodontal bone resorption (8,9). Complex interactions among the various inflammatory mediators and tissue modeling may be involved in the pathogenic mechanisms of periodontitis.

Polyphenolic compounds, including a large class of flavonoids, are enriched in certain vegetables, fruits, seeds and beverages, and are regarded as a class of semi-essential nutrients for humans (10). Dietary intake rich in these compounds is suggested to improve the health of individuals. The beneficial effects of flavonoids have been attributed to their antioxidant, anti-inflammatory, anticancer and gastro-, cardiovascular- and oral-protective properties (11–13). Quercetin (3,5,7,3′, 4′-pentahydroxyflavone) is an abundant flavonol-type flavonoid. The effects of quercetin on a variety of inflammatory processes and immune functions have been reviewed (14–19). In this study, the effect of quercetin on experimental periodontitis, induced by LPS injection and silk ligation, was examined *in vivo* in a rat model.

Material and methods

Experimental design

To examine the effect of osteoclast formation induced by LPS on dental alveoli, three groups ($n = 4$ per group) of 6-wk-old male Sprague–Dawley rats, weighing 180–230 g, were injected daily with 10 μL of 0, 1 or 5 mg/mL LPS (*Escherichia coli* serotype 055:B5; Sigma Co., St Louis, MO, USA) dissolved in phosphate-buffered saline, at the palatal gingiva around the first and second molars for 3 d. Five days after the final injection, all animals were killed by carbon dioxide inhalation. The right palatal specimens (including gingivae, teeth and bones) around the molars were taken and fixed in 4% paraformaldehyde. After EDTA decalcification, dehydration and paraffin embedding, the palatal specimens were sectioned buccopalatally into 4-μm-thick sections and prepared for hematoxylin and eosin (H&E) and tartrate-resistant acid phosphatase (TRAP) staining (20,21). In each specimen, five sections were selected for recording the number of osteoclasts on the palatal bony surface within the distance from the root surface to the bone concavity of palatal vessels. The osteoclasts were identified with red staining near to the bony resorptive lacunae and multiple nuclei (≥ three).

To further evaluate the effect of quercetin on osteoclast formation induced by LPS, 21 Sprague–Dawley rats were randomly divided into three groups (LPS, quercetin and LPS-plus-quercetin groups), with seven animals in each group. Rats in the LPS group were injected daily with 5 mg/mL LPS at the palatal gingiva on the right side, as described above, and given oral feeding with dimethyl sulfoxide (DMSO; 1 mL/kg body weight; Mallinckrodt Baker, Phillipsburg, NJ, USA) daily from 1 d before the start of the experiment. Animals in the quercetin group were given oral daily doses of quercetin (75 mg/kg body weight (22,23); Alfa Aesar, Ward Hill, MA, USA) dissolved in DMSO at a concentration of 75 mg/mL from 1 d before the experiment. Rats in the LPS-plus-quercetin group received both treatments. The oral dosing was performed using an intragastric metal tube without anaesthesia. After 5 d, all animals were killed by carbon dioxide inhalation, and palatal specimens were taken and prepared for histology.

Finally, the effect of quercetin on the bony level of the dental alveolar crest after the ligation was evaluated. Thirty-six male Sprague–Dawley rats were randomly divided into three groups. Rats in the nonligation group received no ligation, whereas 3-O silk (diameter; 0.200 mm) (surgical silk sutures; UNIK, Taipei, Taiwan) was wrapped bilaterally around the cervical margins and knotted at the mesiobuccal side of the maxillary second and mandibular first molars in the ligation group. Rats in these two groups were fed daily with DMSO. Rats in the ligation-plus-quercetin group were given the same silk ligations as rats in the ligation group, and also received daily doses of quercetin (75 mg/kg in DMSO) from 1 d before the ligation. As quercetin was dissolved in DMSO, all rats in nonligation and ligation groups were dosed daily with the solvent as in the ligation-plus-quercetin group. On days 0, 4, 8 and 12, three animals from each group were killed by carbon dioxide inhalation. Maxillary and mandibular specimens were taken and fixed in 4% paraformaldehyde and prepared for microcomputerized tomography (micro-CT) and histology.

In the present study, all animals were kept in a 12 h–12 h light–dark cycle and maintained on standard laboratory rat chow and tap water *ad libitum*. All experiments were conducted in accordance with guidelines for the welfare of experimental animals and were approved by an Institutional Animal Care and Use Committee, National Defense Medical Center, Taipei, Taiwan.

Microcomputed tomography (micro-CT) imaging

All jaw biopsies were subjected to micro-CT imaging using a multimodality preclinical imaging system (FLEX *Triumph*; Gamma Medica-Ideas, Northridge, CA, USA) equipped with a CT sub-system. The X-ray tube was operated at an accelerated potential of 75 kVp (kVp, kilovolts peak, is the maximum voltage across an X-ray tube) with a beam current of 120 μA. The field of view for micro-CT was fixed at 61.44 mm leading to ×2 magnification of images. The micro-CT images were taken under fly mode, with 1024 projections and one frame per projection to achieve a voxel size of 120 μm × 120 μm × 120 μm. Micro-CT data were acquired and reconstructed using Triumph XO software (Gamma Medica-Ideas) and then visualized and analysed using VIVID software (Gamma Medica-Ideas). This enabled us to observe the morphology around the tooth and dental alveolar bone in all dimensions, including the cemento-enamel junction (CEJ), root

surface and dental alveolar crest, as well as the relationships among these areas. Micro-CT with reconstructed three-dimensional images was also used to assess the distance between the CEJ and the coronal level of the alveolar bone crests (the micro-CT bone levels) at 12 sites, including the mesio- and distobuccal sites, the mesio- and distopalatal sites of the right and left maxillary second molars, and the distobuccal and distolingual sites of the right and left first mandibular molars.

After the micro-CT scanning, the maxillary specimens on day 8 were prepared for histology. On the mesial surfaces of the second molars in each rat, the following histometric measurements were performed: the distance of the CEJ to the coronal level of epithelial cells (JEc; attachment loss); the distance of the CEJ to the alveolar bone crest (ABC; the alveolar crest bone level); the distance of the apical level of epithelial cells (JEa) to the ABC (the CT attachment); and the area of inflammatory cell-infiltrated connective tissue (ICT; Fig. 1). The area of ICT was measured in a zone of 0.14 mm^2 of subepithelial gingiva on the mesial surface of the maxillary second molar in each rat, as in previous studies (24,25). In brief, a grid point intersection analysis was used to estimate the areas of infiltrated and total connective tissue of interdental gingiva at ×120 magnification.

Statistical analysis

Repeated-measures analysis of variance (ANOVA) was used to evaluate the influence of quercetin or ligation treatment (the between-subject factor), as well as the observation interval, the examined location at the maxillary or mandibular molars, the right or left side, and the buccal or palatal/lingual site (the within-subject factors) on the location of dental alveolar bone crest measured by micro-CT. One-way ANOVA, with Duncan's test for *post hoc* analysis, was used to determine the effect of LPS on osteoclast formation, and the effect of quercetin on the induced osteoclast formation and histometric measurements after ligation. a p-value < 0.05 was considered significant.

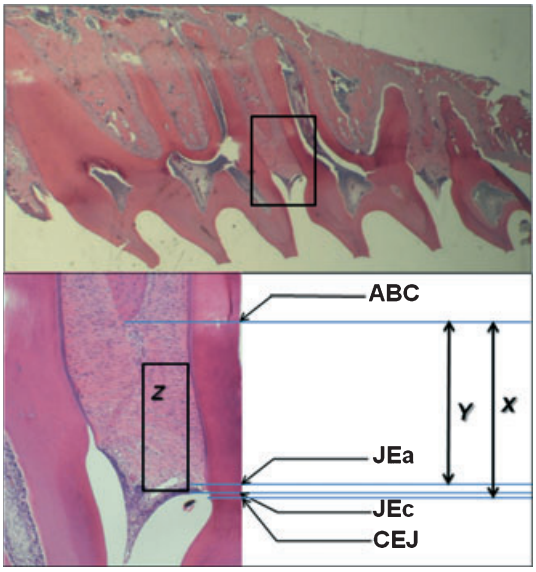

Fig. 1. Periodontal histometric measurements performed at the mesial surface of the maxillary second molar in rats. The top micrograph shows the histology of the molar region from a control rat. A higher magnification vies of the boxed region in that micrograph is shown below. Histometric measurements, including the attachment loss (CEJ to JEc), the alveolar crest bone level (CEJ to ABC = *X*), the connective tissue attachment (JEa to ABC = *Y*), and the area of inflammatory cell-infiltrated connective tissue (ICT = *Z*) in a zone of 0.14 mm^2 of subepithelial gingiva (the boxed zone in the lower micrograph), were performed. Abbreviations: CEJ, cemento-enamel junction; JEc, the coronal level of epithelial cells; JEa, the apical level of epithelial cells; and ABC, the coronal level of alveolar bone crest.

Results

A dose-dependent increase in osteoclast numbers on the palatal surfaces of dental alveoli was observed in rats given LPS concentrations ranging from 0 to 5 mg/mL (Fig. 2A,C). Many osteoclasts with resorptive lacunae were easily observed on the surface of dental alveoli in rats that received 5 mg/mL LPS, whereas bone formation was observed on the palatal surfaces of dental alveoli in rats that received no LPS (Fig. 2). Treatment with quercetin (75 mg/kg) reduced the number of osteoclasts induced by LPS (Fig. 2B,D).

Using micro-CT, apically located alveolar bone crests at ligation sites in rats from the ligation group were generally observed, whereas the same sites in rats from the nonligation group had no apically located alveolar bone crests. Apically located bone crests were rebounded in rats from the quercetin-plus-ligation group (Fig. 3A). On the buccal surface of the right maxillary second molars, the mesial bone levels (CEJ-ABC) at the four observation time points of 0, 4, 8 and 12 d were similar in the nonligation group (Fig. 3B); however, mesial bone levels on days 8 and 12 were greater compared with those on days 0 and 4 in the ligation group. For animals in the ligation-plus-quercetin group, mesial bone levels on days 8 and 12 were lower than those in the ligation group and greater than those in the nonligation group. Similar results were repeatedly observed at the other 11 sites measured (Fig. 3B). By the repeated-measures ANOVA, the mean bone levels observed with micro-CT were significantly different between the treatment groups, the observation intervals, the right and left sides and the buccal and palatal/lingual locations (Fig. 3C).

Significant differences in the dental alveolar bone level, ICT area, attachment loss and CT attachment among

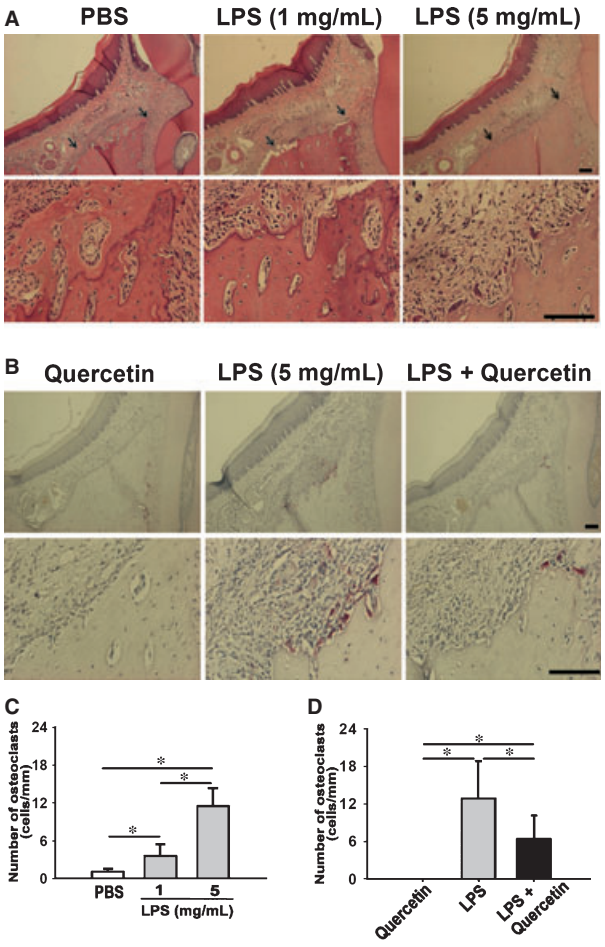

Fig. 2. Effect of quercetin on lipopolysaccharide (LPS)-induced osteoclast formation. (A) The two rows of micrographs represent the palatal histology around the maxillary first molars of rats 5 d after the final injection of 0, 1 or 5 mg/mL LPS dissolved in phosphate-buffered saline (hematoxylin and eosin staining). (B) The micrographs represent the dental alveoli of rats from the quercetin, LPS (5 mg/mL) and LPS-plus-quercetin groups (TRAP staining). The scale bars on the micrographs in A and B represent 50 μm, and arrows indicate the bony surfaces for the osteoclast observation. (C) The effect of LPS injections on osteoclast formation ($n = 4$ per group; $^*p < 0.05$). (D) A comparison of osteoclast formation among the three animal groups ($n = 7$; $^*p < 0.05$).

the three groups were observed by histometry (Fig. 4). *Post hoc* analysis further revealed that the alveolar crest bone levels and ICT areas in the ligation-plus-quercetin group were significantly lower than those of the ligation group and significantly greater than those of the nonligation group. Attachment loss (CEJ to JEc) in the ligation and the ligation-plus-quercetin groups was significantly greater than that in the nonligation group. The mean CT attachment in the ligation group was significantly greater than that in the nonligation and the ligation-plus-quercetin groups, whereas CT attachment differed between the nonligation and the ligation-plus-quercetin groups.

Discussion

In this study, we demonstrated an ameliorative effect of quercetin on the bony destruction resulting from experimental periodontitis in rats. Quercetin is a major flavonoid and forms the backbone for many other flavonoids. It is likely that the bioactivity of quercetin is underestimated because it continues to be shown to have a large spectrum of biological effects (15). Quercetin is thought to be a candidate for preventing various diseases because of its anti-inflammatory effects (23,26–28). Here, we provide *in vivo* evidence that quercetin may have a preventive potential in inflammatory periodontal diseases. In this study, periodontal destruction was induced by LPS injections (Fig. 2) and silk ligation (Figs 3 and 4) in rats. Like previous experiments, osteoclastic bone resorption and periodontal destruction could be induced (7,29–31).

The development of periodontitis involves complex mechanisms associated with bacteria and immune modulations; therefore, the experimental periodontitis we induced in rats in this study has certain limitations (32). The LPS injection, for instance, is a simplification of the disease mechanisms (33,34), whereas periodontitis induced by ligation causes unnatural plaque retention and unavoidable trauma to the local gingival tissue (35). In the present experiments, nevertheless, LPS-induced osteoclast formation occurred in a dose-dependent manner, and the formative pattern on the surface of dental alveolar bone in rats that received phosphate-buffered saline (no LPS) shifted to a resorptive pattern, with osteoclasts accompanied by resorptive lacuna, in rats that received 5 mg/mL LPS. In rats that received 1 mg/mL LPS, bone formation and bone resorption were observed. Quercetin treatment significantly reduced osteoclast formation induced by 5 mg/mL LPS injections. Detail of the mechanisms of the ameliorative effect of quercetin on the periodontal destruction is still unknown; however, LPS could induce inflammation and tissue damage through the induction of cytokines, such as interleukin-1, tumor necrosis factor or interleukin-6 (36), and these cytokines could further induce the production of secondary mediators, resulting in amplification of the inflammatory response and leading to the destruction of connective tissue and osteoclastic bone resorption (37). Recent studies have demonstrated that the flavonoids, including quercetin and luteolin, inhibit or block the inflammatory mediators induced by LPS from periodontal pathogens (38,39). A

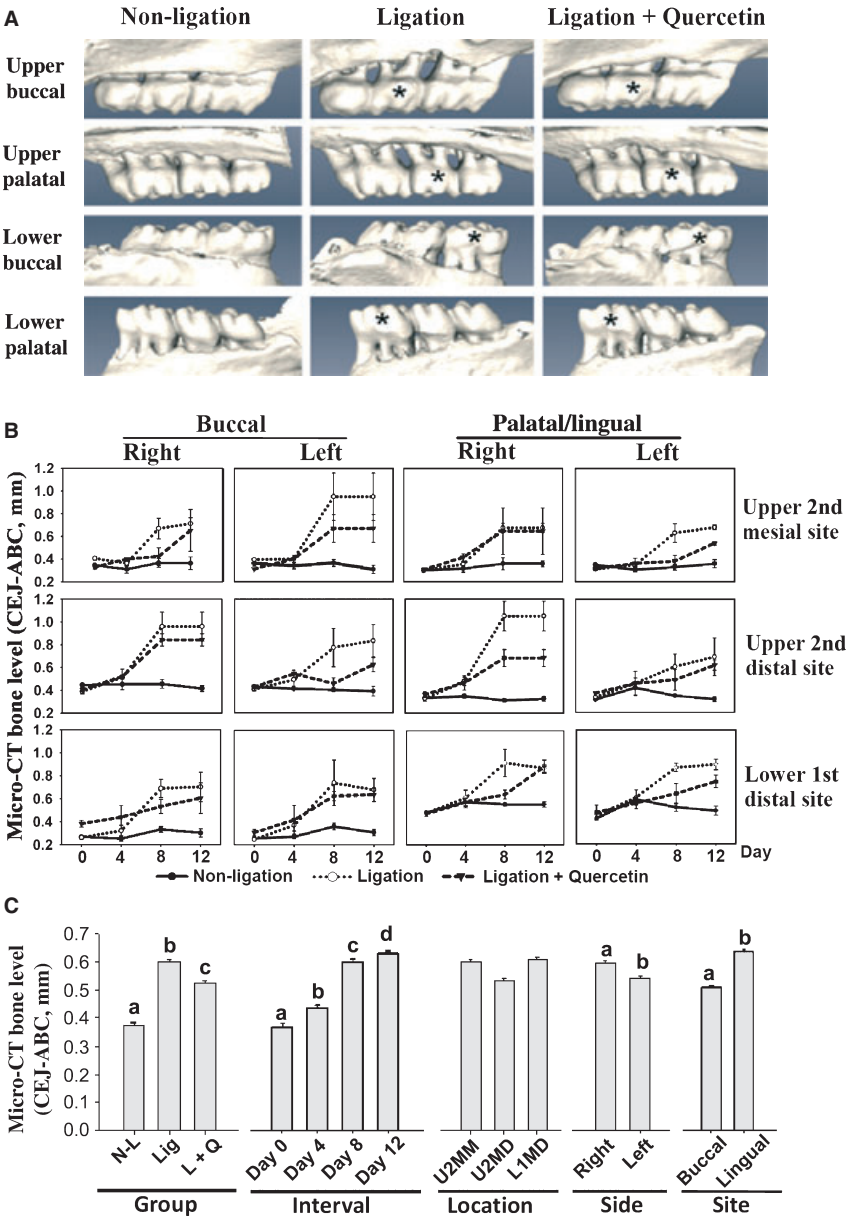

Fig. 3. Micro-CT bone levels among the nonligation, ligation and ligation-plus-quercetin groups. (A) Reconstructed three-dimensional CT images for maxillae (the first and second rows) and mandibles (the third and fourth rows) viewed from the buccal and palatal/lingual direction in the three groups of rats on experimental day 12 (asterisks indicate the molars with ligation). (B) Bone levels for the 12 sites from micro-CT images examined at the four observation time points of 0, 4, 8 and 12 d. (C) The influence of the ligation or quercetin treatment (the between-subject factor), the observation interval, the location examined at the maxillary or mandibular molars, the right or left side, and the buccal or palatal/lingual site (the within-subject factors) on bone levels revealed by micro-CT with a repeated measures analysis of variance (a, b, c and d; the subgroups by *post hoc* analysis when $p < 0.05$ obtained). Abbreviations: N-L, nonligation group; Lig, ligation group; L + Q, ligation-plus-quercetin group; U2MM, the maxillary second molar mesial site; U2MD, the maxillary second molar distal site; and L1MD, the mandibular first molar distal site.

suppressive effect of quercetin on bone resorption has been observed in co-cultures of mouse spleen and ST2 cells, and in cultures of osteoclast progenitor cells (40). This is the first study to show an ameliorative effect of quercetin on experimental periodontal destruction, demonstrated *in vivo* using a rat model.

In this study, experimental periodontitis in rats was induced by silk ligation around the tooth neck to retain bacteria. The destruction of dental alveolar bone was evaluated by micro-CT and histology. By micro-CT, the bone levels at ligation sites and adjacent sites were more apical than that at the sites away from the ligation (Fig. 3). For example, increased micro-CT bone levels were found at the distal sites of the maxillary first molar (the

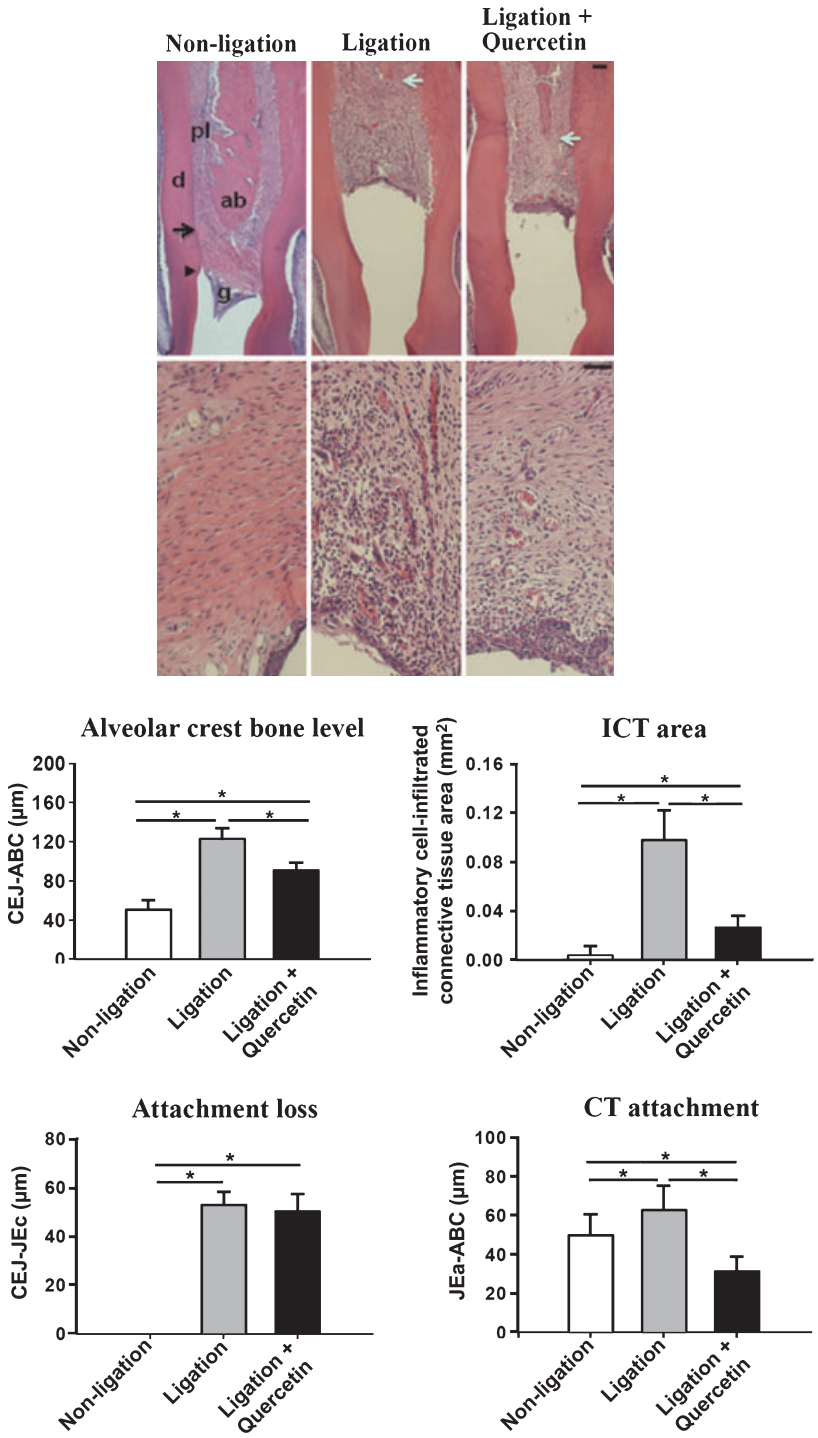

Fig. 4. Histological and histometric observations of maxillary intermolar tissue in nonligation, ligation and ligation-plus-quercetin groups. Histographs in the first row show histological images, including in dentin (d), gingiva (g), alveolar bone (ab) and periodontal ligament (pl). Arrowhead indicates the level of CEJ, arrow indicates the level of alveolar bone crest, and white arrows indicate the crest of dental alveolar bone. The micrographs in the second row represent higher magnifications of images in the first row. Hematoxylin and eosin staining; scale bars represent 50 μm. Comparisons of alveolar crest bone level, attachment loss, CT attachment and ICT areas among the three animal groups are summarized in the graphs below.

site adjacent to ligation) and the maxillary second molar (a ligation site) compared with those at the distal site of the maxillary third molar (the site away from ligation). The micro-CT bone levels at the sites away from the ligation were similar among the three animal groups. Among the three animal groups, the mean micro-CT bone

levels in those sites related to ligation were significantly different (Fig. 3C); however, the levels also showed a time-dependent increase during the 12 d observation. The levels were more apical on lingual sides if compared with those on buccal sides; however, uneven aleveolar bony levels on the right and left sides were recorded. The unequal placement of ligation on the right and left sides, by a right-handed operator, for example, might partly explain the uneven results on the two sides, although the exact reason is still unknown. By histometry, the alveolar crest bone level and ICT area were significantly reduced in the ligation-plus-quercetin group compared with the ligation group, and significantly greater compared with the nonligation group (Fig. 4). However, the data showed a similar attachment loss in the ligation and the ligation-plus-quercetin groups. Moreover, an increased CT attachment in the ligation group was observed when compared with the nonligation and the ligation-plus-quercetin groups. These data indicate that micro-CT findings might be useful in determining the dental alveolar bone level (or loss); however, detailed evaluation of microanatomy in the periodontium by histology is still needed.

In the present study, we found that quercetin reduced LPS-induced osteoclast formation and ligation-enhanced alveolar bone loss. Although the exact mechanism or pathway for amelioration is unknown, our histometric data show that a reduction of ICT area and alveolar bone level occurred in the ligation-plus-quercetin group and the ligation group. Moreover, a narrower CT attachment was observed in the ligation-plus-quercetin group compared with the other two groups (it was longest in the ligation group). This might indicate that quercetin has an anti-inflammatory effect on the induced periodontal inflammation, which may result in consequent dental alveolar bone loss. In the ligation and ligation-plus-quercetin groups, the soft tissue destruction occurred due to the direct and indirect damage resulting from the ligation; however, the bone loss was indirectly caused by the induced inflammation. In the quercetin group, inflammation was induced, but not severe enough to make an obvious bone loss, which might lead to a narrower CT attachment in the ligation-plus-quercetin group if compared with the two other groups. In the present study, the attachment loss (CEJ to JEc) in the ligation and the ligation-plus-quercetin groups was significantly greater than that in the nonligation group, which suggests that quercetin might not prevent attachment loss and might suggest that the beneficial effects of quercetin are questionable. However, the present histological findings were restricted to a short duration of 8 d, which might not be long enough to observe the effect of quercetin on the long-term attachment loss in the chronic disease of periodontitis. We assume that as long as alveolar bone loss can be prevented during periodontitis, CT attachment will be re-established after the infective etiology is removed. Moreover, unavoidable trauma from the ligation itself is a limitation of ligature-induced experimental periodontitis. Further detailed studies are therefore needed, such as those involving a longer observation period (the chronic pattern of disease), follow-up of the healing response of CT attachment after ligature removal, an animal model with naturally occurring periodontitis (no trauma from ligation) or infection with periodontal pathogens (as well as LPS).

In conclusion, we have demonstrated that quercetin treatment reduced LPS-induced osteoclast formation and ligature-enhanced periodontal inflammation and alveolar bone loss. Moreover, quercetin decreased ICT areas and narrowed CT attachment, but did not affect attachment loss in the present study. Therefore, we suggest that quercetin may have an ameliorative effect on periodontal tissue inflammation, which may reduce bone loss.

Acknowledgements

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