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ORIGINAL ARTICLE

Curcumin inhibits inflammatory response and bone loss during experimental periodontitis in rats

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Abstract

Objective. Curcumin, an active ingredient of turmeric, is proved to be a potential candidate of controlling inflammation and bone resorption, but few reports are on the periodontitis. The purpose of this study was to evaluate whether the intragastric administration of curcumin could inhibit the inflammation and alveolar bone resorption in rats following ligature-induced experimental periodontitis. ***Materials and method.*** Male Wistar rats were randomly divided into three groups: no ligature placement and administration of vehicle, ligature placement and administration of vehicle, ligature placement and administration of curcumin. After the animals were sacrificed, their mandibles were collected for morphological, histological and immunohistochemical analysis; their gingival tissues were collected for cytokine measurements. ***Results.*** Bone resorption was significantly higher in the experimental periodontitis animals treated with vehicle compared with the curcumin-treated group or the control group. Furthermore, receptor activator of nuclear factor-κB ligand (RANKL), receptor activator of nuclear factor-κB (RANK), osteoprotegerin (OPG), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) expression levels were higher in the experimental periodontitis animals treated with vehicle compared with the curcumin treated group or the control group. ***Conclusions.*** Curcumin may decrease alveolar bone loss in the experimental periodontitis rats via suppressing the expression of RANKL/RANK/OPG and its anti-inflammatory properties.

Key Words: *curcumin, experimental periodontitis, bone resorption, inflammation*

Introduction

Periodontitis is a chronic infectious inflammatory disease characterized by the damage of the tooth-supporting structures, the pathogens of which are bacteria of dental plaque [1,2]. Recently, a consensus states that the actual tissue destruction occurring in periodontitis is mostly relying on host-response inflammatory mechanisms that strives to eradicate the dental plaque from the gingival sulcus, but less on pathogen-mediated mechanisms [3]. These periodontal pathogens release a number of potential virulence factors to stimulate host immune response to produce various inflammatory molecules, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) [3,4], then leads to gingivitis, periodontal pocket formation and alveolar bone resorption until tooth loss [4,5].

Inflammation arising from bacterial infection has been known to exacerbate bone destruction [6]. Alveolar bone loss acts as the cardinal pathological and clinical characteristics of periodontitis. Bone is a dynamic organ that is continuously remodeled by the balance between bone resorption via osteoclasts and bone formation via osteoblasts, which is closely regulated by the receptor activator of nuclear factor-κB ligand (RANKL) and its decoy receptor, osteoprotegerin (OPG) [7]. RANKL, expressed by osteoblasts, fibroblasts, activated T cells and B cells [4], acknowledges its responsibility for the differentiation and maturation of osteoclast precursor cells into active osteoclasts; while OPG, a soluble tumor

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necrosis factor receptor-like molecule, acts as a decoy receptor which binds to RANKL and inhibits osteoclast development. As a matter of fact, based on pre-clinical animal studies and preliminary human clinical studies, the RANKL system plays an important role in the treatment of destructive periodontal disease and other bone resorption-related diseases [8,9].

Much effort has been made to develop effective drugs for prevention and treatment of bone resorption. Recently, active compounds endowed with a capacity to modulate the host inflammatory response and bone resorption have been concerned as they may represent potential new therapeutic agents for treating periodontitis [10].

Curcumin is derived from the root of the turmeric plant Curcuma longa and possesses anti-oxidant, anti-tumor and anti-inflammatory properties [11,12]. Preventive as well as therapeutic anti-inflammatory effects of curcumin treatment have been observed in several researches, such as *Helicobacter pylori*-infected gastritis mouse model [13], latex-sensitized allergic mouse pulmonitis [14] and human osteoarthritis clinical study [15]. Studies involving systemic administration of curcumin have shown its beneficial effects on a variety of inflammatory conditions by several signaling molecules, including nuclear factor-κB (NF-κB) [16], TNF-α [17], activator protein-1 [18], IL-6 [19] and interleukin-1β (IL-1β) [20]. Furthermore, the potential effects of curcumin on the bone diseases have been reported that bone loss is decreased via inhibiting the c-fos and c-jun expression [21]. Bharti et al. [22] also demonstrated that curcumin can inhibit the osteoclast precursors to differentiate into osteoclasts via suppressing RANKL-induced NF-κB activation.

Although a range of biological and pharmacological activities of curcumin are reported [20,23], few studies have been done to investigate the roles of curcumin in periodontitis. Recently Guimarães et al. [24,25] demonstrated the potent anti-inflammatory activity of curcumin in experimental periodontitis rat models. In our previous study, we showed that *in vitro* curcumin inhibits the gene and protein expressions of inflammatory cytokines TNF-α and IL-1β in RAW264.7 cells (mouse macrophage cell line) treated with lipopolysaccharide (LPS) [26]. These *in vitro* advantages render curcumin to be considered as a promising anti-inflammatory candidate for periodontitis therapy. The present study was designed to evaluate whether the curcumin could control the inflammation and prevent the alveolar bone resorption in animal models of periodontitis. The evaluation was based on the modulation of RANKL system and the expression of TNF-α, IL-6 in the periodontal tissues.

Materials and methods

Materials

Curcumin was bought from Sigma-Aldrich Co. (St. Louis, MO). Mouse TNF-α and IL-6 Enzyme Linked Immunosorbent Assay (ELISA) Kit were purchased from R&D systems (Minneapolis, MN). Primary antibodies for RANKL, receptor activator of nuclear factor-κB (RANK), OPG were obtained from Santa Cruz Biotechnology Inc. (USA). The radioimmunoprecipitation assay (RIPA) lysis buffer and bicinchoninic acid (BCA) assay for gingiva protein extraction and concentration measurement were obtained from Thermo Fisher Scientific (Rockford, IL). Other agents used here were locally available.

Animals

Thirty male Wistar rats (200–250 g) were bought from the Experimental Animal Center of Wuhan University. The animals were kept in metal cages with access to standard rat food and tap water *ad libitum* in a temperature-controlled room. Prior to the surgical procedures, all animals were allowed to acclimatize to the laboratory environment for 1 week. All experimental procedures were conducted in accordance with the guiding principles of the Animal Care and Use Committee in the School of Stomatology, Wuhan University.

Experiment design

For experimental periodontitis induction, a sterilized 3-0 black braided nylon thread (Surgilon; USS/DG, Norwalk, CT) ligature was placed around the cervix of both mandible first molars of rats anesthetized with 10% chloral hydrate (400 mg/kg, i.p.) as described elsewhere [27]. The ligature was knotted on the mesial in order to make sure the ligature stayed there for the whole experimental period. The animals were randomly divided into three groups: no ligature placement and administration of saline ($n = 10$); ligature placement and administration of vehicle ($n = 10$), ligature placement and administration of curcumin ($n = 10$). Curcumin was administered daily in a volume of 100 mg/kg for 30 days. The solutions were freshly prepared just before intra-gastric administration and this dose was decided according to a previous study [28]. Twenty-four hours after the 30 days' administration [29] animals were sacrificed.

Measurements of mandible bone loss

The animals were sacrificed under deep anesthesia and the right mandibles were dissected. The gingival tissues were removed and stored at $-80°$C until further analysis. Then the right mandibles were fixed in 10% buffered neutral formalin for 48 h, briefly

washed in running tap water, defleshed and stained with aqueous methylene blue (1%) in order to differentiate bone from teeth. The bone loss was measured through the distance from the amelo-cemental junction to the alveolar crest (ACJ-AC) as described previously [30]. Six points were made in the long axes of both buccal and lingual root surfaces for each first molar: the average of the six heights was used as a measure of alveolar bone loss for each molar (expressed in mm). Quantitative analyses were performed individually by two trained and calibrated examiners who were blind to the groups.

Cytokine measurements

The total protein was extracted from the gingival tissues stored at −80°C using the RIPA lysis buffer, then the concentration was determined by the BCA assay according to the manufacturer's protocol using bovine serum albumin as a standard. The TNF-α and IL-6 concentrations were evaluated using the ELISA kit according to the instructions. The ratios of TNF-α and IL-6 concentrations compared with the total protein concentration represented the TNF-α and IL-6 expression levels in gingival tissues.

Histology and immunohistochemistry analysis

The left mandibles of the sacrificed animals were dissected, fixed in 10% buffered neutral formalin for 2 days, decalcified in a 10% EDTA solution for 6 weeks at 4°C and embedded in paraffin. Each sample was sliced into 5 μm sections in mesio-distal directions. Continuous sections, which contained the mesial root, distal root and the furcation area between the two roots, were stained with the hematoxylin and eosin (HE), alveolar bone loss areas in the furcation regions were measured by SPOT RT software, v3.5 (Spot Diagnostic Instruments, Sterling Heights, MI) in three random HE-stained sections [31].

Five-micrometer sections were used for immunohistochemical analysis of RANKL, RANK and OPG. The sections were rehydrated, the endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min and the non-specific binding was blocked by non-immunogen animal serum for 30 min at room temperature. The RANKL, RANK, OPG polyclonal antibodies 1:80 were applied, incubated overnight at 4°C and the immunohistochemical second antibody kit (SP kit, Maixin, Fuzhou, China) was used according to the manufacturer's manual. The sections were visualized using 3, 3′diaminobenzidine (DAB) and counterstained with hematoxylin. The negative controls represented the omission of the primary antibodies.

Three representative sections of each specimen were analyzed under a light microscope. Relative staining intensity was assessed for each antibody, scored as follows: 0 = no immunoreactivity; 1 = weak, but visible staining; 2 = moderate staining; and 3 = strong staining intensity [29]. All quantitative evaluations for the HE-stained and immunohistochemical sections were performed individually by two trained and calibrated examiners who were blind to the groups.

Statistical analysis

ACJ-AC distances, bone resorption areas in furcation region and pro-inflammatory cytokine expression levels were analyzed with ANOVA, followed by Bonferroni's test. Immunohistochemical staining of RANKL, RANK and OPG expression levels were analyzed with Kruskal–Wallis analysis. Kappa tests were used for examiner calibration (all indexes in the present study for Kappa tests >0.90).

Results

Curcumin decreases ACJ-AC distance in experimental periodontitis rats

A microscopic examination of the mandibles at 30 days after the ligature placement revealed increased ACJ-AC distances in the lower first molar regions in the experimental periodontitis animals (Figures 1B and C), which demonstrated that ligature-induced experimental periodontitis significantly increased bone loss in rats when compared to the control group (Figure 1A). Figure 1G showed average ACJ-AC distances of the three group animals at the end of the study. All the experimental periodontitis animals, subjected to vehicle or curcumin, showed significant ($p < 0.05$) elevation in ACJ-AC distances compared with the control group and the curcumin-treated animals showed less elevation ($p < 0.05$) in ACJ-AC distances compared with the vehicle-treated group.

Curcumin decreases bone resorption areas in furcation region in experimental periodontitis rats

Inflammation infiltrated the gingival tissue and led to the destruction of periodontal supporting tissues. To gain insights into the severity of bone resorption in the furcation regions, HE staining sections were performed and analyzed. Consistent with the increased ACJ-AC distances as described above, similar results of the bone resorption areas were obtained in the furcation regions in experimental periodontitis animals (Figures 1E and F) when compared to the control group (Figure 1D). Experimental periodontitis animals, subjected to vehicle or curcumin, had significant ($p < 0.05$) elevation of bone resorption areas in the furcation regions compared with the control group and curcumin inhibited ($p < 0.05$) the increase of bone resorption areas in furcation regions compared with the vehicle-treated group (Figure 1H).

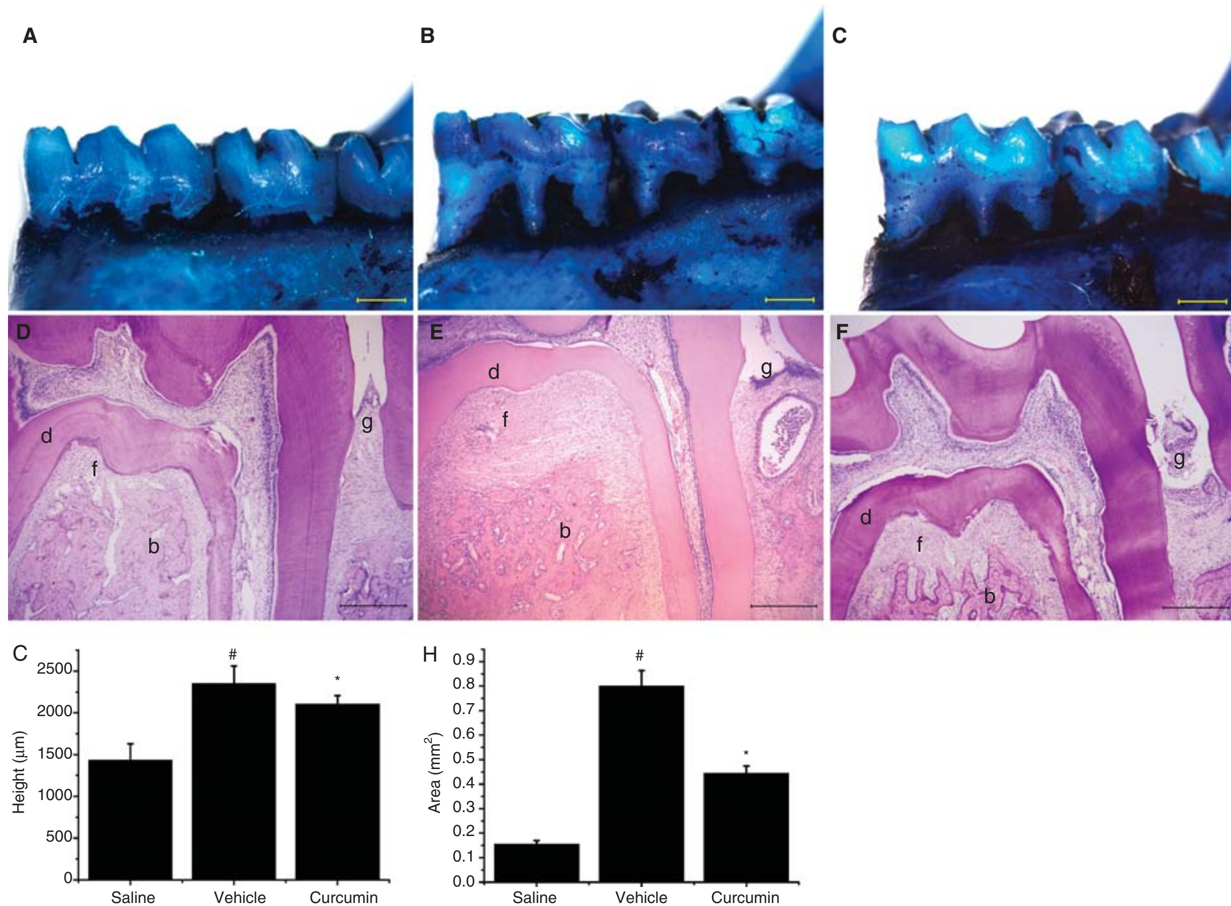

Figure 1. Microscopic aspects of the ACJ-AC distance. (A) Control animals. (B) Mandibles from experimental periodontitis animals treated with vehicle showed severe bone resorption and root exposure. (C) Moderate bone resorption and root exposure were observed in the curcumin treated animals. Bar in (A-C): 800um. Histological aspects of the bone resorption areas in root furcation region (HE staining). (D) Control animals. (E) Experimental periodontitis group treated with vehicle showed increased bone loss. (F) Mild bone loss was observed in the curcumin treated animals. Bar in (D-F): 400um. b = alveolar bone, d = dentin, f = furcation region, g = gingival papilla. Significant increase of the ACJ-AC distance (G) and bone loss areas in the furcation regions (H) were observed in experimental periodontitis animals treated with vehicle compared with control animals, and curcumin treatment reduced the increase of ACJ-AC distance and bone loss areas in experimental periodontitis animals. Data shown are mean ± SD of 10 animals per group. For both ACJ-AC distance (G) and bone loss areas in the furcation regions (H): #p<0.05 compared with control group; *p<0.05 compared with control group; #& *p<0.05 there was difference between vehicle treated group and curcumin treated group.

Effect of curcumin on the expression level of RANKL, RANK and OPG in experimental periodontitis rats

To study the molecular mechanism of bone resorption during experimental periodontitis progression, immunohistochemical staining sections for RANKL, RANK and OPG were performed. No staining was observed in the furcation area of control animals for RANKL (Figure 2A), RANK (Figure 2D) and OPG (Figure 2G). Experimental periodontitis animals treated with vehicle showed moderate staining for RANKL (Figure 2B) and OPG (Figure 2H); strong staining for RANK (Figure 2E). Experimental periodontitis animals treated with curcumin presented very weak staining for RANKL (Figure 2C), RANK (Figure 2F) and OPG (Figure 2I). According to the quantification of immunohistochemical staining intensity, it showed that the RANKL ($p < 0.05$), RANK ($p < 0.05$) and OPG ($p < 0.05$) expression levels were higher in the experimental periodontitis animals treated with vehicle, compared with the curcumin-treated group or the control group; so was the experimental periodontitis animals treated with curcumin compared with the control group (Figures 2M–O). The positive yellow or brown staining grains were right in the furcation regions where the bone resorption occurred; the negative controls were not stained (Figures 2J–L).

Effect of curcumin on the expression level of TNF-α and IL-6 in the gingival tissues of experimental periodontitis rats

To investigate the expression levels of pro-inflammatory cytokines TNF-α and IL-6 in the gingival tissues of experimental rats, concentrations of TNF-α and IL-6 in the gingival tissues were obtained using the ELISA assay. Ratios of the pro-inflammatory cytokines

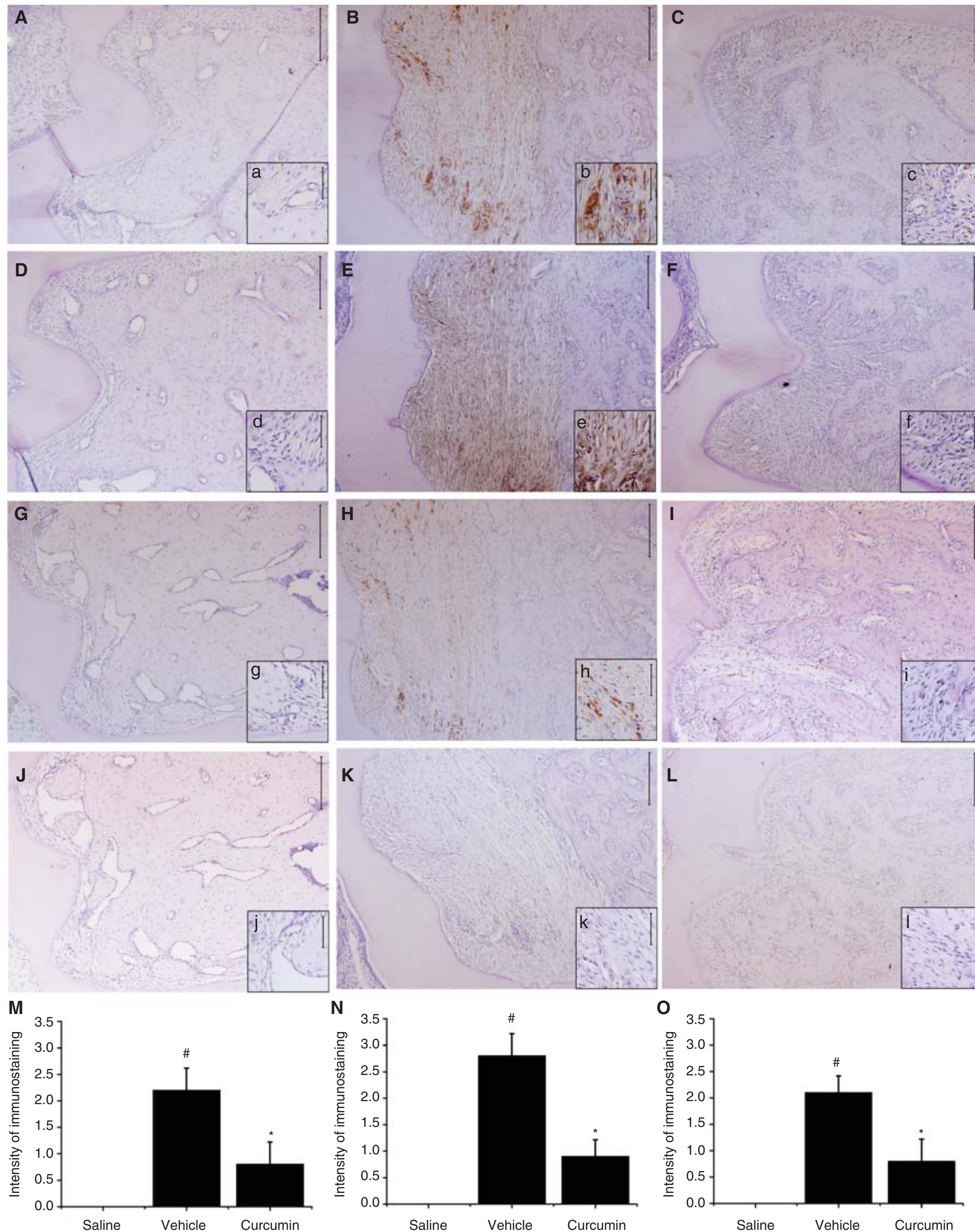

Figure 2. Immunohistochemical staining for RANKL/RANK/OPG. No staining for RANKL (A), RANK (D), OPG (G) was observed in the furcation area of control animals. Moderate staining for RANKL (B) and OPG (H), strong staining for RANK (E) in experimental periodontitis animals treated with vehicle. Very weak staining for RANKL (C), RANK (F), OPG (I) in curcumin treated animals. (J-L) Negative control staining was performed via omitting exposure to the primary antibodies. Bar in (A-L): 200um. Bar in (a-l): 60um. The intensity of immunohistochemical staining was assessed to evaluate RANKL (M), RANK (N), OPG (O) expression level in the furcation area. Experimental periodontitis animals, subjected to vehicle or curcumin, had higher expression level of RANKL/RANK/OPG compared with the control animals, and curcumin treatment decreased the expression of RANKL/RANK/OPG in the experimental periodontitis animals. Data shown are mean ± SD of the average staining of all sections analyzed per area (n=10 per group). For all RANKL (M), RANK (N), OPG (O): #p<0.05 compared to control group; *p<0.05 compared to control group; #& *p<0.05 there was difference between vehicle treated group and curcumin treated group.

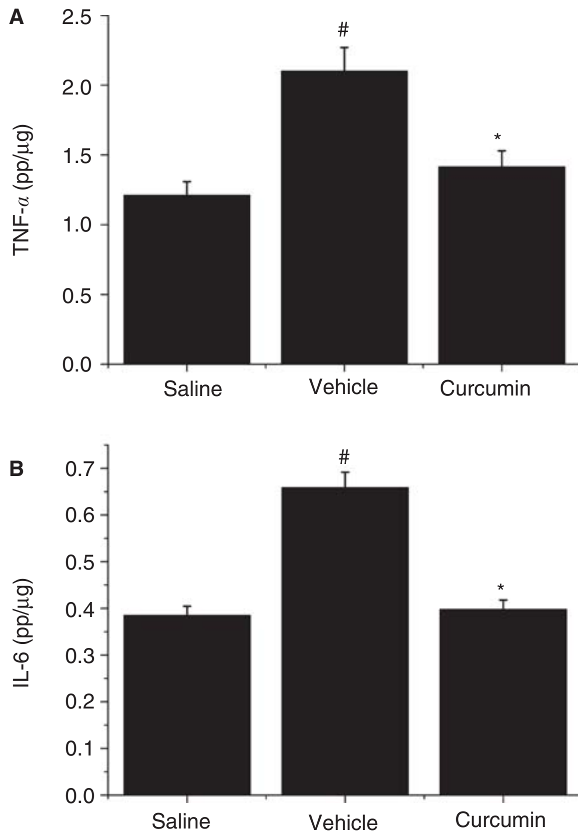

Figure 3. Effect of curcumin on cytokine release in gingival tissues. TNF-a (A) and IL-6 (B) expression levels were higher in the experimental periodontitis animals treated with vehicle compared with the curcumin treated group or the control group, but no difference was found between the latter two. Data are presented as means (pg/ug)±SD of ratios of each cytokine's concentration compared with the total protein concentration in gingival tissues (n=10 per group). For both TNF-a (A) and IL-6 (B): #p<0.05 compared to control group; *p>0.05 compared to control group; #& *p<0.05 there was difference between vehicle treated group and curcumin treated group.

TNF-α and IL-6 concentrations compared with the total protein concentration represented their expression levels in the gingival tissues. As shown in Figures 3A and B, TNF-α ($p < 0.05$) and IL-6 ($p < 0.05$) expression levels were higher in the experimental periodontitis animals treated with vehicle, compared with the curcumin-treated group or the control group, but no difference ($p > 0.05$) was found between the experimental periodontitis animals treated with curcumin and the control group.

Discussion

It is well established that the host inflammatory immune response against pathogens results in the periodontal tissues' destruction [3,4]. Alveolar bone resorption and inflammation are hallmarks of periodontitis. The development of novel drugs that can inhibit bone loss offers opportunities to target not only soft tissue inflammation but also the destructive bone loss observed in periodontitis [32]. Curcumin is a representative polyphenolic compound found in the dietary spice turmeric [33], responsible for suppression of osteoclastogenesis [22]. Ligature-induced rat experimental periodontitis offers a reliable model with site-specific, time-dependent alveolar bone resorption. Therefore, we investigated the effects of curcumin on the alveolar bone resorption in a ligature-induced experimental periodontitis model and found that curcumin could decrease the ACJ-AC distances and alveolar bone loss areas of lower first molars in the experimental periodontitis rats. These results are consistent with the inhibition effects of curcumin on bone loss in the distal femur of diabetes rats [21]; and inconsistent to the finding that curcumin could not prevent the decrease of bone volume fraction [24]. The inconsistency may be explained by the different bone loss assessment method and longer experimental period.

The RANKL/RANK interaction has an important role in the pathogenesis of periodontitis [32]. RANKL binding to the receptor RANK, present on the surfaces of osteoclast precursor cells, promotes their maturation and activation; whereas OPG combines RANKL with high affinity as a decoy receptor competitively inhibiting the RANKL/RANK interaction and interrupts the osteoclastogenesis [34]. To date, no direct effect of curcumin on the RANKL/RANK system has been reported in the periodontitis in the literature. For the first time, our results showed that RANKL, RANK expression levels were lower in the experimental periodontitis rats submitted to curcumin compared with the vehicle group, which might illuminate that curcumin decreased alveolar bone loss in the experimental periodontitis rats, at least in part, via reduction of the osteoclast regulators' (RANKL/RANK) expression. It has been reported that curcumin suppresses osteoclastogenesis in bone marrow stromal cells by decreasing RANKL expression [35]. Other studies also demonstrate that drugs inhibiting RANKL expression reduce the inflammation and alveolar bone loss in a *P. gingivalis*-induced periodontitis mice model [36] and a ligature-induced periodontitis rat model [29]. All these findings support our deduction that curcumin may decrease alveolar bone loss in the ligature-induced periodontitis rats via inhibiting RANKL expression. Our experiment also found that OPG expression went down in the curcumin exposure group accompanying the decrease of RANKL expression. It may attribute to that decreasing RANKL expression likely needs less bone protector OPG to counteract with.

Among a wide range of biological and biochemical activities, curcumin therapeutic effects are being investigated in anti-inflammatory disease, such as inflammatory pancreas and ileum [19,28]. Recent research on the analog of curcumin finds it can inhibit various inflammatory mediators such as monocyte chemotactic protein-1, interleukin-10, TNF-α and

IL-6 in LPS-induced RAW 264.7 and U937 cells (human macrophage cell line) [37]. Other studies also indicate that curcumin can modulate the inflammatory response, suppress TNF-α and IL-6 in a ligature-induced periodontitis rat model [24] and LPS-induced periodontitis rat model [25]. In accordance with these results, we found that the inflammatory cytokines TNF-α and IL-6 had lower expression levels in the experimental periodontitis rats submitted to curcumin compared with the vehicle-treated group. TNF-α and IL-6, associated with inflammatory cell migration and osteoclastogenesis processes, are the first pro-inflammatory cytokines involved in the periodontal inflammation and up-regulate other classic pro-inflammatory immunity cytokines [3]. TNF-α either increases the osteoclast activity via a RANKL system or directly stimulates pre-osteoclasts to differentiate into mature osteoclasts [38], while IL-6 indirectly enhances the osteoclastic bone resorption via stimulating the expression of RANKL and interleukin-1 by osteoblasts [39,40]. Therefore, curcumin might alleviate inflammatory responses and alveolar bone loss in the experimental periodontitis rats by inhibition of the expression of pro-inflammatory cytokines TNF-α and IL-6, synergistically or independently working with a RANKL system.

In conclusion, we provide evidence that curcumin can decrease the inflammatory responses and alveolar bone loss in ligature-induced experimental periodontitis rats via decreasing pro-inflammatory cytokines (TNF-α and IL-6) and osteoclastogenesis related molecules (RANKL and RANK). Curcumin may provide periodontists a complementary approach to the conventional periodontal therapy. However, future studies are needed to further elucidate the mechanisms of curcumin on anti-inflammation and suppressing alveolar bone loss in periodontitis.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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