Original Communication

Effects of Curcumin on Alveolar Bone Loss in Experimental Periodontitis in Rats: A Morphometric and Histopathologic Study

Aysun Akpinar[1], Metin Calisir[2], Nebi Cansın Karakan[3], Aysan Lektemur Alpan[4], Fahrettin Goze[5], and Omer Poyraz[6]

[1] Cumhuriyet University, Faculty Of Dentistry Periodontology Department, Sivas, Turkey
[2] Adıyaman University, Faculty Of Dentistry Periodontology Department, Adıyaman, Turkey
[3] Afyonkarahisar Saglik Bilimleri University, Faculty of Dentistry, Periodontology Department, Afyonkarahisar, Turkey
[4] Pamukkale University, Faculty of Dentistry, Periodontology Department, Denizli, Turkey
[5] Cumhuriyet University, Faculty Of Medicine, Pathology Department, Sivas, Turkey
[6] Cumhuriyet University, Faculty Of Medicine, Microbiology Department, Sivas, Turkey

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Abstract: *Background:* Curcumin is found in the rhizomes of the turmeric plant that has been showed antioxidant and anti-inflammatory effect. The aim of this study was to evaluate the effects of systemic curcumin therapy on alveolar bone loss in an experimental periodontitis model in rats. Material and Methods: Thirty-two male Wistar rats were randomly divided to 4 groups: 75 mg/kg/daily curcumin (C75; *n* =8), 150 mg/kg/daily curcumin (C150; *n* =8), Control (*n* =8), and Ligated (*n* =8). Curcumin was administrated using gastric gavage. After 12 days, the rats were sacrificed. Right mandibles samples were histopathologically examined. Alveolar bone loss was measured. Interleukin 1β (IL-1β) and interleukin 10 (IL-10) were evaluated in the serum samples and gingival homogenates. *Results:* The measurements of alveolar bone loss in the mandibular molars revealed significantly higher bone-loss values in the Ligated group than the Control, C75 and C150 groups. The IL-1β levels in the gingival homogenates were significantly increased in the Ligated group compared to those of the Control, C75 and C150 groups. The serum IL-1β levels in the Ligated group were significantly higher than the Control group. The mean osteoblast numbers in the Ligated group were lower than those of the Control, C75 and C150 groups. The C150 groups showed significantly more osteoblasts than the Control group. The osteoclast numbers in the Ligated group increased significantly compared to the C75, C150 and control groups. *Conclusion:* This study demonstrates that systemic administration of curcumin at the 75 and 150mg/kg doses reduced alveolar bone loss in the periodontal disease in rats.

Keywords: Alveolar bone loss, Antioxidant, Curcumin, Ligature-induced, Histomorphometric, Micronutrition

Introduction

Periodontal disease is a common infection that is considered to be initiated by microorganisms in the dental plaque biofilm [1]. Periodontitis is a chronic inflammatory disease characterized by gingival inflammation and resorption of alveolar bone [2]. Periodontal disease is the result of complex inter-relationships between infectious agents and host factors [3], and is modulated through multiple factors including genetics, smoking, general health, social variables and diet. Mechanical and surgical therapy with or without antibacterial therapy applications are common treatment modalities for periodontal disease, but host modulation strategies with many agents have been popular in recent years [4, 5]. Some reagents may have both antimicrobial and anti-inflammatory properties and, therefore, might be useful for controlling and treating multiple aspects of periodontal disease pathology [6]. A loss of homeostatic balance between reactive oxygen species (ROS) and the antioxidant defense systems that protect and repair vital tissue, cell and molecular components are believed to be responsible for periodontal destruction [7]. The term oxidative stress refers to serious imbalance between the production of reactive species and antioxidant defenses. Antioxidants are molecules in the body that protect the cells from ROS by removing the oxidants or repairing the damage caused by the ROS in vivo [8].

Curcumin is found in the rhizomes of the turmeric plant (Curcuma longa L.), which belong to the ginger family [9]. More recently, it has been used by the food industry as an

additive, a flavoring, a preservative, and a coloring agent with its orange-yellow color being used in mustard, margarine, soft drinks, and beverages. Curcumin use in dentistry has only been reported in recent years [10, 11]. The studies are limited to the effect of curcumin to the on alveolar bone healing. The underlying effects of curcumin have been investigated both in vivo and in vitro regarding the following situations: transcription factors such as nuclear factor-κB regulation (NF-κB) [12] and activated protein-1 (AP-1) [13]; growth factors such as vascular endothelial cell growth factor (VEGF) and fibroblast growth factor (FGF) [14, 15], enzymes such as cyclooxygenase-2 (COX-2), inducible nitric oxide synthesis (iNOS), and matrixmetalloproteinases (MMP) [16–19]; inflammatory cytokines [20, 21]; protein kinases [22, 23]; and intercelular adhesion molecules such as cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (ELAM-1) [24, 25].

Interleukins play a crucial role in periodontal pathogenesis. Interleukin 1(IL-1) is a pro- inflammatory cytokine and has two important forms: interleukin 1 beta (IL-1β) and interleukin 1 alpha (IL-1α). IL-1 levels are elevated in both tissues and gingival crevicular fluid from active periodontally destructive periods compared to healthy sides. IL-1β is an important mediator of inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, apoptosis and osteoclastogenesis [26]. Previous studies have shown that curcumin suppressed pro-inflammatory mediators significantly, including interferon-γ, tumor necrosis factor-alpha (TNF-α) and IL-1β by inhibiting NF-κB activation and reducing oxidized glutathione [27–29]. Interleukin 10 (IL-10) is a potent anti-inflammatory cytokine that suppresses both immunoproliferative and inflammatory responses although it can also downregulate the synthesis of proinflammatory cytokines and chemokines, such as IL-1, interleukin-6 (IL-6), and TNF-α. Some studies have shown that osteoclast formation is inhibited by IL-10 [30, 31].

Curcumin, a selective phosphorylase kinase inhibitor, is a naturally occurring phytochemical in turmeric [32]. In an experimental model of chronic liver injury, curcumin increased the IL-10 level in hepatic cells and had an anti-inflammatory effect [33]. Similarly in bronchoalveolar lavage fluid, curcumin increased the IL-10 level via the interleukin 17A (IL-17A) level recovered from bronchoalveolar lavage fluid. This decreased the experimentally induced allergic reactions observed with asthma [34]. Colonic mucosal biopsies and colonic myofibroblasts with active inflammatory bowel disease taken from children and adults were cultured ex vivo with curcumin. As a result IL-1β levels decreased and IL-10 levels increased. p38 mitogen activated protein kinase activity was suppressed by curcumin [35]. A strong relationship between IL-10 levels and curcumin extracts and its fractions was observed [36].

The effects of curcumin on IL-10 are contradictory. Some studies have mentioned that it has no positive effects or has no impact, in addition to mentioning some negative effects [33, 37, 38]. Due to its efficacy and regulation of multiple targets, curcumin exhibits potential antioxidant, anti-inflammatory, antiviral, antibacterial, antifungal, and anticancer activities against various malignant diseases, diabetes, allergies, arthritis, Alzheimer's disease, and other chronic illnesses [39–45].

Considering its anti-inflammatory and antioxidant properties and its capability to significantly inhibit the expression of proinflammatory cytokines, curcumin play a significant role in preventing the initiation and progression of periodontitis. The aim of this study was to analyze the effects of systemic curcumin therapy on alveolar bone loss, both morphometrically and histopathologically in a ligature-induced periodontitis rat model.

Material and Methods

Animals and Treatment

Thirty-two male Wistar rats weighing 250 ± 10 g were used in the study. The animals were kept in temperature-controlled cages (approximately 25 0C); and exposed to a 24 h light–dark cycle of equal lengths, and they were free to drink water and eat food ad libitum. The experimental procedure was approved by the Animal Ethics Committee of Cumhuriyet University School of Medicine (document number: 65202830/175). The rats were randomly divided into four groups:

- 75 mg/kg/daily curcumin (C75; n=8),
- 150 mg/kg/daily curcumin (C150; n=8),
- Non-ligated (Control; n=8)
- Ligated control (Ligated control; n=8).

General anesthesia was administered using ketamine (Eczacıbasi Ilac Sanayi, Istanbul, Turkey) (40 mg/kg). In order to induce experimental periodontitis, a 4–0 silk suture (Dogsan Sanayi, Istanbul, Turkey) was placed subgingivally, around the gingival margin of the right mandibular first molars of the rats outside of the Control group. In the C75 and C150 groups, curcumin (Sigma-Aldrich, Saint Louis, MO, USA) which had a chemical structure 1,7-bis(4-Hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione was administered systemically with a vehicle solution (physiological saline) using a gastric gavage, at doses of 75 mg/kg/d and 150 mg/kg/d, respectively. To create a sham effect, saline solution was administrated us-

ing gastric gavage on both the Control and Ligated groups. Daily systemic treatment with curcumin was continued for 11 days; all of the rats were killed on day 12. All of the animals sacrificed by using cardiac punction. Blood was taken and immediately centrifuged at 3000 rpm for 10 minutes to obtain serum samples which were stored at -20›C in Eppendorf tubes until used for enzyme-linked immunosorbent assay (ELISA) studies. Gingival biopsy samples were obtained, rinsed thoroughly in sterile saline and stored at -80°C until ELISA analysis. The right mandibles were resected, fixed in 10% neutral-buffered solution and stained for histomorphometric analysis.

Measurement of Alveolar Bone Loss

Soft tissues from the right mandibular region were defleshed manually and then cleaned. The jaws were washed, dried and embedded into 1% aqueous methylene blue solution to identify the cement-enamel junction (CEJ) level. The photographs were taken with a stereomicroscope (x25) and transferred to a computer. The alveolar bone loss was measured by a single examiner who was masked from the samples' identities. At three different sites in the right mandibular molar region by recording the distance from the CEJ to the top of the alveolar bone crest using Vision Image Analysis software (Clemex, Quebec, CANADA). Mean values of measurements were calculated for statistical analysis. (Figure 1)

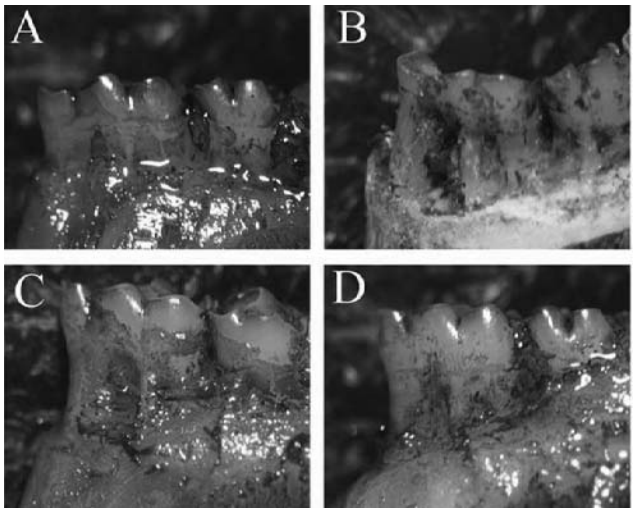

Figure 1. Images of alveolar bone loss in groups. (A) Control, (B) Ligated group, (C) 75mg/kg/daily curcumin (C75) and (D) 150 mg/kg/daily curcumin (C150).

Laboratory assays

The gingival tissues collected from rats were homogenized and processed as described by Safieh-Garabedian et al. [46]. Levels of IL-1β (detection range: 31.3–2,000 pg/ml; sensibility or lower limit of detection: 4 pg/ml of recombinant rat IL-1β) and IL-10 (detection range: 15.6–1000 pg/ml; sensibility or lower limit of detection: <5 pg/ml of recombinant rat IL-10) serum and the gingival samples (samples per group) were determined by using commercial ELISA kit (Invtrogen, Camarillo, CA). ELISA analysis was performed according to the manufacturer's recommendations, using 96-well plates presoaked with appropriate antibodies. 50 µl standard diluents buffer was added to each 50µl sample. 50µl of biotin conjugate was added to each sample and incubated for 2 h at 370C room temperature. After further incubation at RT for 2 h, the plates were washed 4 times, and 100 µl of streptavidin HRP (diluted 1:5000) was added at RT for 30 minutes. The plates were aspirated and washed for four times. 100 µl of stabilized chromogen was added and incubated for 30 minutes. 100 µl stop solution was added and the absorbance of the color at 450 nm is measured. The resulting values were expressed in pg/ml.

Histopathological and Histomorphometric analysis

Mandible sections were fixed in 10% formalin 24 h, than soaked into 10% formic acid to demineralize the sample. Then the specimens were dehydrated and embedded in paraffin, and 6-µm thick sections were obtained along the molars in a mesio-distal plane for hematoxylin and eosin and Masson's trichrome staining as described by Toker et al. [6]. Alveolar bone and interdental septum were analyzed under light microscopy (Eclipse E 600, Nikon, Tokyo, Japan). The inflammatory cell infiltration (ICI) of the periodontal tissues was scored using the following scale; ICI not visible (score=0); slightly visible ICI (score=1); and dense visible ICI (score=2). A semi quantitative scoring method was used for determining osteblastic activity; no activity (0); mild/ moderate activity (1); and high activity (2). Osteoclasts, which are large cells with multiple nuclei near the border of the resorption surface were counted morphologically in the histological assessment [47]. (Figure 2).

Statistical analysis

Statistical analyses were performed with SPSS 14.0 for Windows (SPSS, Inc., Chicago, IL, USA). Kolmogorov-Smirnov test was performed to determine the data distribution. The data were not disturb normally, thus nonparametric tests were performed. The four groups were compared using the Kruskal-Wallis test. Two independent

group comparisons were performed using the Mann-Whitney U test. The data are presented as mean ± standard deviation and $p<0.05$ was considered statistically significant. The ratios of the presence of ICI and osteoblastic activity were analyzed using a chi-square test.

Results

Eight rats from each group completed the study. None of the rats developed any systemic conditions during the study. The presence of the silk ligature around the mandibular first molar induced an inflammatory reaction and periodontitis in the tissue. The measurements of alveolar bone loss in the mandibular molars revealed significantly higher bone-loss values in the Ligated control group than in the Control, C75 and C150 groups, respectively ($p<0.05$). (Figure 3).

The levels of the pro-inflammatory cytokine IL-1β in the gingival homogenates were significantly increased in the Ligated control the group, compared to those of the Control, C75 and C150 groups, respectively ($p<0.05$). (Figure 4) The serum IL-1β levels in the Ligated group were significantly higher than in the Control group ($p<0.05$). (Figure 5)

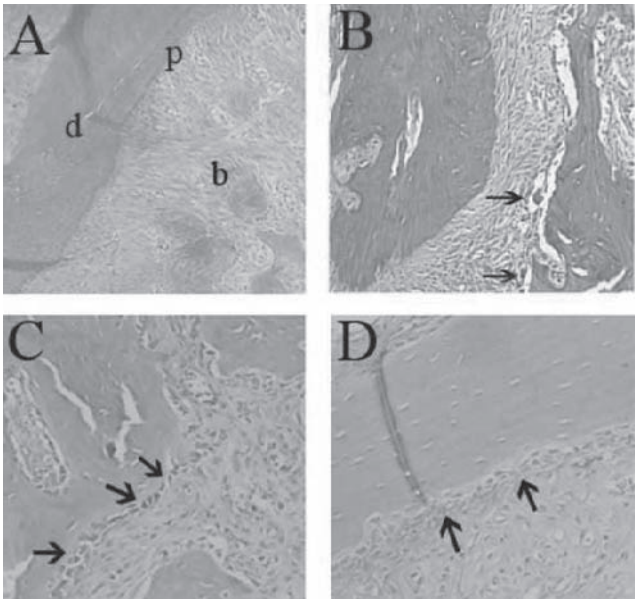

Figure 2. Histopathological images of all groups. (A) Control group. Normal periodontium; alveolar bone (b), periodontal ligament (p), and dentin (d). (B) Ligated group. A large number of osteoclasts are seen on the alveolar bone surface. (C) 75 mg/kg/daily curcumin (C75) group. New bone formation is observed (arrows). (D) 150 mg/kg/daily curcumin (C150) group. Osteoblasts, lined up along the alveolar bone, are seen (arrows). (Hematoxylin and eosin; original magnification: A, X10; B, X10; C,X10; D, X10)

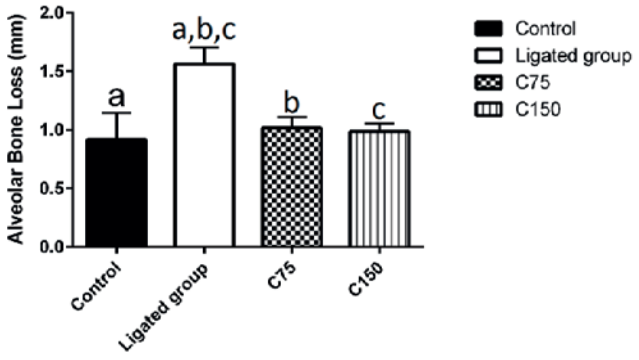

Figure 3. Mean alveolar bone loss in columns with standard deviations in bars of groups. a: Alveolar bone loss in the Ligated group was significantly higher than the Control group ($p<0.05$); b: Alveolar bone loss in the Ligated group was significantly higher than the 75mg/kg/daily curcumin (C75) group ($p<0.05$); c: In the Ligated group alveolar bone loss was significantly higher than the 150 mg/kg/daily curcumin (C150) group ($p<0.05$).

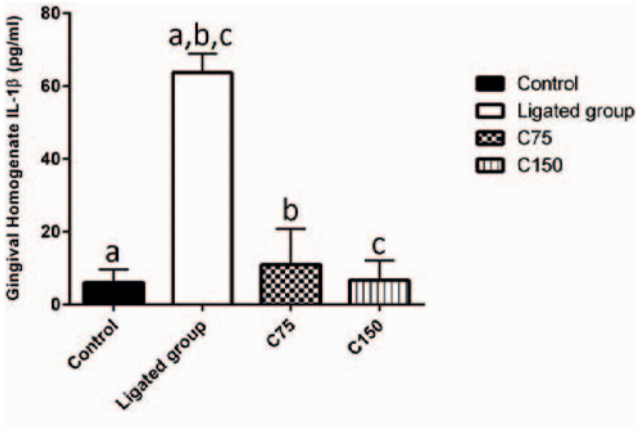

Figure 4. IL-1β levels in gingival homogenates groups (columns are mean values and bars are standard deviation values). a; In the Ligated group, IL-1β levels significantly higher than the control group ($p<0.05$). b; In the Ligated group, IL-1β levels significantly higher than the 75 mg/kg/daily curcumin (C75) and c; 150 mg/kg/daily curcumin C150 group ($p<0.05$)

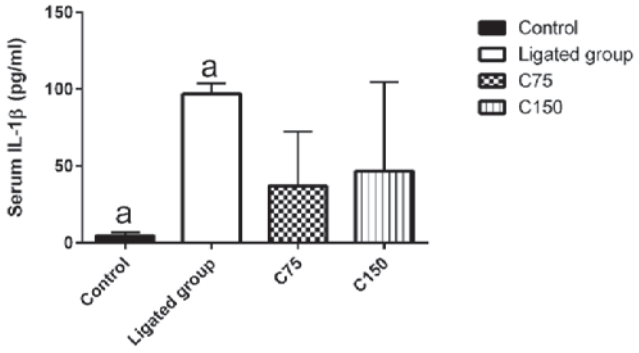

Figure 5. Serum IL-1β levels of groups (columns are mean values and bars are standard deviation values).a; Ligated group revealed significantly higher IL-1β level than Control group ($p<0.05$)

Table 1. Mean osteoblast and osteoclast numbers in groups.

	Control	Ligated control	C75	C150
	mean±SD	mean±SD	mean±SD	mean±SD
Osteoblast number	18.50±5.24	14.42±6.70†	34.66±14.06	38.16±17.80‡
Osteoclast number	1.66±0.81	19.28±10.01*	5.33±4.92	5.33±3.98

† Osteoblast numbers in the Ligated group revealed lower numbers than compared to the Control, C75 and C150 groups, respectively ($p < 0.05$); ‡ C150 groups showed significantly higher osteoblast numbers than the Control group ($p<0.05$); *Osteoclast numbers in the Ligated group were significantly higher than the Control, C75 and C150 groups, respectively ($p < 0.05$).

The serum and gingival homogenates IL-10 levels of the Ligated control, Control, C75 and C150 groups were below the minimal detection limit of the assay kit in thus the data is not shown.

The Ligated group revealed less osteoblastic activity than in the Control, C75 and C150 groups, respectively ($p<0.05$). The C150 group showed significantly more osteoblasts than the Control group ($p<0.05$). The osteoclast numbers in the Ligated group were significantly increased compared to the Control, C75 and C150 groups ($p<0.05$). (Table 1)

Discussion

The understanding of the causation and pathogenesis of periodontal disease has changed dramatically thus the approach toward periodontal treatment has evolved over 30 years from blocking inflammation to moderating it [46]. Many studies in the literature refer to links between periodontal disease and dietary incomes [48–50]. Nutrients derived from one's diet can act as antioxidants, co-enzymes in energy production and metabolic processes, as well as components of tissue structures that keep the body functioning properly and help to maintain good overall health, including oral health [51].

Functional groups of curcumin reveal different chemical structures. Three common anologs of curcumin include: diferuloylmethane, demethoxycurcumin and bisdemethoxycurcumin. There are different antioxidant activities of these three forms. As well as it has been reported that variety in the chemical structures of curcumin are responsible for different biological and pharmacological activities of these common forms [52]. In our study we selected the diferuloylmethane form because of its better antioxidant activity that has been reported in literature [53, 54]. NF-κB act ivat ion plays a crucial role in periodontal pathogenesis. IL-6, TNF, receptor activator levels of nuclear factor-κB (RAN K) and nuclear factor-κB ligan d (RANKL) are known to be regulated by NF-κB, TN F an d RANK L are potent activators of NF-κB . Blocking NF-κB pathway inhibits alveolar bone loss.

Diferuloylmethane has an inhibitory effect on NF-κB pathway by various mechanisms such as activating caspases 9,7 and 3 [55], inhibition TNF dependent NF-κB activation [56].

Curcumin has been used as a dietary supplement, and has been widely used in Ayurvedic medicine for its anti-oxidant, anti-inflammatory, analgesic and antimalarial properties. Zhou et al. [57] evaluated the effects of curcumin, on ligature-induced experimental periodontitis and suggested that bone loss in subjects treated with systemic curcumin therapy was significantly lower than in the vehicle group. However the receptor activator levels of RANKL, RANK, osteoprotegerin (OPG) levels, TNF-α levels and IL-6 expression levels were higher in the animals treated with the vehicle, compared with the curcumin treated group and the control group. The results of our study were in accordance with another study [58] in which chemically modified curcumin decreased alveolar bone loss based on both morphometric and μ-CT analyses and served as a potent inhibitor due to its inflammatory and collagen-destructive mediators.

Bone remodeling is characterized by mineralized matrix removal, which is when bone that is old or damaged by osteoclasts is subsequently replaced by new bone formed by osteoblasts. The osteoclast differentiation is mainly regulated by RANKL. RANKL activates its receptor, RANK which is then expressed on osteoclasts. Thereby, several intracellular signaling pathways occur via RANKL-mediated RANK stimulation; hence, bone resorption occurs. ROS may play a role as a secondary messenger or a modulator in transducing signals at low levels [59]. Several antioxidants may inhibit RANKL-mediated active oxygen species generation. Therefore, the signal pathways required for osteoclastogenesis is suppressed by these antioxidants [60]. It has been reported that several antioxidants such as curcumin, coenzyme Q10 and selenium may inhibit reactive oxygen species that are necessary a signaling inter-

mediates for osteoclast differentiation; osteoclastogenesis could thereby be controlled by antioxidants. Curcumin has been reported to inhibit osteoclastogenesis [61]. Moreover, curcumin inhibited RANKL-mediated osteoclast differentiation by suppressing NF-kB, which causes signal some-associated IkB kinase inhibition [62]. Curcumin has prevented bone loss in ovariectomized rats and has decreased osteoclastogenesis by reducing RANKL activity due to its antioxidant activity [63]. Also oxidative stress observed in patients with periodontitis could be linked to inflammation biomarkers such as C-reactive protein (CRP) [64]. NF-kB pathway regulates CRP levels. Although some studies have indicated potent inhibitory activities of curcumin on NF-kB activation and some oncogenes expressions like Akt, CDKs, MAPKs and iNOS [65]. With all these effects curcumin can prevent both inflammatory response and bone loss that observed in periodontitis.

According to a study performed by Guimarães M.R. [66], curcumin did not prevent alveolar bone resorption, but its anti-inflammatory effects on pro-inflammatory cytokines showed that curcumin has a potential effect on periodontal inflammation. In our study we found that curcumin had significantly decreased the progression of alveolar bone loss at two different dosages (75 and 150 mg/kg).

Many experimental periodontitis models have been applied on rats such as using ligature on molar teeth, dietary manipulation, and the introduction of pathogenic microorganisms [58, 67]. The placement of a ligature around the molar teeth of rats leads to the appearance of inflammation beneath the ligature [68] in one study, the most intense bone loss was achieved by day 11 after ligature placement [69]. Periodontitis and gingival inflammation occurred at the first molars in all of the ligated rats at 11th day which are ligated then rats were sacrificed on the 12th day in our study.

IL-1β and IL-10 have very important roles in the pathogenesis of periodontitis, through their involvement in regulating the host's inflammatory response and bone resorption. Elevated IL-1β levels in gingival crevicular fluid have been associated with periodontitis severity [70, 71]. The central functions of IL-10 family cytokines converge on protecting organs and tissues from damage by inhibiting the innate and adaptive immune responses of leukocytes and limiting the potential tissue damage caused by inflammation [72]. We determined that IL-10 and IL-1β not only in serum samples but also in gingival homogenates could eliminate the systemic inflammation factor that could be developed in rats. We found that gingival homogenate IL-1β levels were significantly lower and serum IL- 1β levels were significantly higher in the curcumin treated groups than in the Control group. In our study we used 75 and 150mg/kg doses applied to rats, no side effects of curcumin were observed during the study. Many studies performed with low doses of curcumin showed efficiency [73, 74], although many studies used a 400 mg/kg curcumin dosage was used in many studies, no side effects were observed in these studies [28, 75]. A more precise optimal dose-setting study for curcumin, to achieve maximum effects with fewer side effects, is needed to in order apply this therapy in a clinical setting [76]. Curcumin has been consumed as a dietary spice at doses of up to 100 mg/day [77]. Clinical trials have shown that humans can tolerate curcumin even at a dose of 8 g/day, although high doses (2–12 g) of curcumin have shown several side-effects [78] with some subjects reporting mild nausea or diarrhea [79]. More recently, curcumin was found to alter iron metabolism by chelating iron and suppressing the protein hepcidin, potentially causing iron deficiency in susceptible patients [43]. Curcumin has poor bioavailability and this limits clinical applications. Main limitations of curcumin are low absorbs ion, rapid metabolism and quick elimination. Dissolution of Curcumin in water is poor and UV can activate photochemical degradation. For increasing bioavailability, several additional methods have been presented [52].

Conclusion

Within its limitations, this study showed that the administration of systemic curcumin with different dosages increased osteoblastic activity and diminished the presence of osteoclasts due to decreased alveolar bone loss in a ligature-induced rat model. Based on our data curcumin can be provided as an additive to periodontal therapy for preventing bone loss. Curcumin is safe, non-toxic and can be used as an adjunct for initial periodontal therapy. Therapotic properties of curcumin put it forward for clinical applications. Further studies should research the relationship between curcumin and ROS inactivation both in-vivo and in-vitro focus on periodontal tissue healing an periodontal regeneration.

Acknowledgments

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Conflict of interest

The authors declare that there are no conflicts of interest

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Metin Calisir
Adiyaman University, Faculty of Dentistry
Department of Periodontology
02140, Adiyaman
Turkey
Tel:+90 416 225 19 20
Fax:+90 416 225 19 21
metincalisir@adiyaman.edu.tr

