

The effect of colchicine on alveolar bone loss in ligature-induced periodontitis

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Abstract: Colchicine is widely used in the treatment of several inflammatory diseases due to its anti-inflammatory effect, but effects on bone metabolism are unclear. The aim of this study was to evaluate the effects of systemically-administered colchicine on healthy periodontium and experimentally-induced periodontitis. In total, 42 male Wistar rats were included in this study. A non-ligated group constituting the negative control group (Control, C, n = 6) and a ligature-only group forming the positive control group (LO, n = 12) were created separately. Twelve rats were treated with 0.4 mg/kg colchicine and another 12 with 1 mg/kg colchicine. In the colchicine-administered groups, right mandibles constituted the ligated groups (1 mgC-L or 0.4 mgC-L) and left mandibles formed the corresponding non-ligated controls (1mgC or 0.4mgC). Silk ligatures were placed at the gingival margin of the lower first molars. The animals were euthanized at different time-points of healing (11 or 30 days). Alveolar bone loss was clinically measured and TRAP+ osteoclasts, osteoblastic activity, and MMP-1 expression were examined histologically. There was no increase in alveolar bone loss with either colchicine dose in healthy periodontium ($p > 0.05$) and the highest level of alveolar bone loss, TRAP+ osteoclast number, and MMP-1 expression were measured in the LO group ($p < 0.05$). The 0.4 mgC-L group showed less alveolar bone loss at 11 days ($p < 0.05$), but greater loss at 30 days. The 1 mgC-L group showed higher osteoblast number than the other ligated groups ($p < 0.05$) at both time-points. In summary, colchicine did not increase alveolar bone loss in healthy periodontium and also may tend to reduce periodontitis progression. However, further extensive study is necessary to understand the mechanism of colchicine action on alveolar bone loss in periodontitis.

Keywords: Periodontitis; Alveolar Bone Loss; Colchicine.

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Introduction

Periodontitis is a chronic inflammatory disease caused by a pathogenic microbiota in the subgingival biofilm. Bacterial challenge induces an inflammatory reaction with an increase in cytokines, prostaglandins, and reactive oxygen species.^{1,2} Sustained inflammation in the tissue progresses into deep tissues and causes loss of supporting connective tissue and

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alveolar bone. Continued inflammation also causes the formation of a periodontal pocket, ultimately leading to tooth loss.³ However, it is now known that the underlying tissue damage observed in this disease results from an excessive immune response to subgingival pathogens.⁴ Therefore, it follows that pharmacological inhibition of host response pathways may be an adjunctive or alternative strategy for treating periodontal disease.⁵

Colchicine, originally extracted from *Colchicum autumnale*, is an anti-inflammatory drug that has been in continuous use for more than 3,000 years.⁶ Colchicine acts by binding tubulin in a poorly reversible manner, forming a colchicine-tubulin complex. At lower doses, this complex interferes with microtubule formation and elongation, and at higher concentrations promotes microtubule depolymerization.⁷ Classically, it was thought that the microtubule disruption caused by colchicine led to anti-inflammatory effects by inhibiting neutrophil chemotaxis, diminishing release of lysosomal enzymes during phagocytosis, inhibiting the expression of adhesion molecules on the surface of endothelial cells and leukocytes and also inhibiting inflammasome activation within macrophages.^{8,9,10,11} In addition, colchicine depresses neutrophil degranulation, which is closely related to the secretory response and oxidative outbreak^{12,13} and colchicine has been shown to attenuate lipid peroxidation and stabilize membranes.¹⁴ Colchicine has also been prescribed for acute gout attacks and prophylaxis, Behcet's Disease¹⁵, familial Mediterranean fever (FMF), pseudo-gout and dermatologic disorders, due to its aforementioned effects.¹⁶

While the anti-inflammatory activity of colchicine is well known, its effect on bone metabolism is unclear. Earlier studies suggested that colchicine decreased osteoclast number and blocked resorption in bones pretreated with parathyroid hormone.¹⁷ Conversely, a recent study reported that prolonged colchicine treatment (1 mg/kg/day for 6 weeks) had a significant negative influence on fracture healing in rat tibia, by inhibiting fracture union and reducing bone strength.¹⁸ In addition, in a recent clinical study that investigated the association between FMF and osteoporosis in adult patients, they found that regular colchicine treatment, which might

have suppressed the inflammatory status of FMF, may not prevent the development of osteoporosis.¹⁹ However, to the best of our knowledge, there are no data regarding the effects of colchicine on alveolar bone loss in periodontitis. Also, whether colchicine can ameliorate or reduce periodontitis progression remains unknown. Consequently, the aim of this study was to investigate the effect of colchicine, which has anti-inflammatory and anti-oxidative effects, on healthy periodontium and alveolar bone resorption in a rat model of experimental periodontitis, both histopathologically and histomorphometrically.

Methodology

Animals and experimental model

This study was approved by the Ethical Committee for Animal Studies of Cumhuriyet University School of Medicine. In total, 42 male Wistar rats (weighing 230–250 g) were used in the experiment. Rats were housed in individual cages in a room with 12 h light-dark cycles, with water and food available *ad libitum*.

This study used a split-mouth design to study ligated groups with or without systemic administration of colchicine, with the experimental conditions on one side, and the control on the other. In each rat, a ligature was placed around their right first molar while the contralateral left first molar did not have a ligature as a colchicine control. The animals were divided into groups as follows:

- a. Non-ligated controls (control) (6 rats);
- b. Ligature-only (LO) group (12 rats);
- c. Colchicine 0.4 mg/kg/day - nonligated (0.4 mgC) group (left side) and ligated (0.4 mgC-L) group (right side) (12 rats);
- d. Colchicine 1 mg/kg/day nonligated (1mgC) group (left side) and ligated (1 mgC-L) group (right side) (12 rats).

Induction of experimental periodontitis

The procedure was carried out under general anesthesia using 40 mg/kg Ketamine hydrochloride. A 4-0 silk suture was sub-marginally placed around the first molars of the right mandibular quadrants. All ligatures were positioned subgingivally and lost or

loose sutures were replaced. All ligature placements were performed by the same operator (A.Y.).

Colchicine was prepared as doses of 0.4 mg/kg and 1 mg/kg in 0.5 mL distilled water and systemically administered by gastric feeding at a rate of 0.5 mL daily until sacrifice. Colchicine doses used by studies were in the range from 0.1 mg/kg to 1 mg/kg. And the doses of colchicine were selected based on the doses studies in previous studies which were shown to be effective.^{20,21,22,23} In colchicine groups, half of the animals of each group were sacrificed at 11 days and the remainder at 30 days. Both of these durations are considered to be a suitable time period to observe periodontal disease course in rats.^{24,25,26,27}

Alveolar bone measurements

After sacrifice, mandibles were harvested and soft tissues around the mandibles were excised. The mandibles were stained with 1% aqueous methylene blue to identify the cemento-enamel junction (CEJ). The distance between the CEJ and the alveolar crest was measured at 16× magnification using digital imaging software (Zeiss, Stemi 2000, Oberkochen, Germany) integrated with a stereomicroscope and camera system. All measurements were performed at six points, three buccal and three lingual surfaces, and a mean value for each tooth was calculated. The morphometric measurement of alveolar bone loss was performed by a single examiner (M.B.T.) who was unaware of the identity of the samples.

Histopathological evaluation

After stereomicroscopic analysis, right and left mandibles were immersed in 10% neutral buffered formalin for 48 h. Decalcification of the mandibles was performed with EDTA solution (10%, for 10 weeks). After decalcification, all samples were dehydrated through an ethanol series and embedded in paraffin, then 5 µm serial sections were prepared and used for hematoxylin and eosin (H&E) and tartrate-resistant acid phosphatase (TRAP) histochemical staining. The stained sections were evaluated by light microscopy (Eclipse E 600; Nikon, Tokyo, Japan).

Cuboid osteoblast cells bordered with osteoid and neighboring periodontal ligament and visible on active bone formation surfaces were considered to be

active osteoblast cells and counted. Inflammatory cell infiltration (ICI) of the periodontal tissue was also evaluated in all sections. Total inflammatory cells in an area of 10,000 µm² (neutrophils, lymphocytes, eosinophils, and macrophage cells) were counted at 1,000× magnification. A single examiner (F.G.) who was also unaware of the identity of the samples, performed all histological evaluations.

TRAP histochemistry

Osteoclast cells were identified by TRAP staining on decalcified sections. The TRAP histochemistry procedure was performed according to Leong's protocol.²⁸ Specimens were rehydrated and treated with a mixture of sodium acetate (0.2 M) and sodium tartrate dibasic dehydrate. Sections were incubated in this solution for 20 min at room temperature. After incubation, fast red TR salt and naphthol AS-MX phosphate were added and a second incubation for 1 h at 37°C was performed. During the second incubation, after the first 30 min, sections were closely monitored under a light microscope. Once the TRAP+ osteoclast cells were stained bright red, the procedure was stopped and sections were washed and stained with Gill's hematoxylin.

MMP-1 Immunohistochemistry

MMP-1 immunohistochemistry was performed in order to evaluate collagen breakdown. After deparaffinization and dehydration of the sections, antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) for 2 h at 70°C. Then the sections were treated with 3% hydrogen peroxide to quench endogenous peroxidase activity. After incubation with normal rabbit serum for 30 min, samples were incubated with primary antibodies overnight. Sections were then washed five times with phosphate buffered saline then incubated with biotinylated immunoglobulin G for 30 min, washed several times with phosphate-buffered saline and reacted with a streptavidin-horseradish peroxidase-conjugated reagent for 30 min. Following three 5-minute washes with phosphate-buffered saline, samples were incubated with AEC chromogen to visualize the immunoreactivity, then counterstained with hematoxylin and analyzed using light microscopy.

Immunohistochemical H-score analysis

Five areas were selected randomly from the sections from each animal to be examined, under a light microscope with 1,000× magnification. Categorical enumeration of the cells within these areas was made according to their immune staining intensity. All cells in an area of 10,000 μm^2 were counted considering the staining intensities. During these counts, both the number of cells showing positive immunoreactivity and the degree of immunoreactive intensity of these cells, as well as the total number of cells which were stained and not stained were considered. No staining was considered as score '0', slight staining intensity was '1', mild staining intensity was '2' and strong staining intensity was '3'. The average of the results of a blind study was taken. To estimate the results of the counts, the H Score formula $[\sum P_i(i+1)]$ was used. In this formula, I represents the staining intensity score and P_i represents the percentage of stained cells.

Statistical analysis

Data are presented as mean \pm SD or percentage as appropriate. Statistical analyses were performed with SPSS® software (vs.23.00). Osteoclast numbers, alveolar bone loss, osteoblast numbers, and MMP-1 H-score were evaluated by the Kruskal-Wallis and Man-Whitney-U tests for pair-wise comparisons. P values < 0.05 were considered statistically significant.

Results

Experimental periodontitis was successfully achieved and ligation caused periodontal destruction and alveolar bone loss around mandibular first molar teeth. The animals did not show any obvious signs of systemic illness throughout the study period and no rats were lost or excluded from the study.

Morphometric analyses

The highest level of alveolar bone loss was measured in the LO group at 11 days ($p < 0.05$) (Figure 1 and 2). There was no significant difference in alveolar bone loss among all control groups. Furthermore, alveolar bone loss was similar in both colchicine groups with a ligature ($p > 0.05$), and

only systemic administration of 0.4 mg colchicine decreased alveolar bone loss ($p < 0.05$).

According to analyses at 30 days, there was no significant difference among all control groups ($p > 0.05$). Although the alveolar bone loss in both colchicine groups was lower than in the L group, the differences were not significant ($p > 0.05$). However, at 30 days, alveolar bone loss was increased in the 0.4 mgC-L group compared to at 11 days ($p < 0.05$).

Histopathological analyses

Figure 3 shows a representative histological view of the groups. The TRAP-positive osteoclast number in the L group was higher than in either of the colchicine groups with a ligature, but the difference was not significant at 11 days ($p > 0.05$) (Figure 4). In addition, the osteoclast number in the L group was higher than in all control groups. The other comparisons showed no significant differences regarding osteoclast numbers at 11 days.

At 30 days, osteoclast numbers in the L and 1mg-L groups were higher than in any of the controls (Figure 3 II-B). Also, the 1mg-L group was found to contain significantly higher osteoclast numbers than the 0.4mg-L group, but the differences were not significant. Among the control groups, there were no significant differences in osteoclast numbers at 30 days, nor were there any significant differences between 11 and 30 days. In contrast, in the L group, there were significant differences between 11 and 30 days in terms of osteoclast number ($p < 0.05$).

ICI was higher in all ligated groups than in the non-ligated controls at either 11 or 30 days ($p < 0.05$). There were no significant differences among all controls in terms of ICI at either time-point ($p > 0.05$). In addition, there were significant differences in ICI between the 0.4 mgC-L and the L group at 11 days. However, at 30 days, there were no statistically-significant differences in ICI among ligated groups.

According to histological analyses, osteoblast numbers were similar among all control groups and no significant difference was observed (Figure 5). Likewise, there were no significant differences in osteoblast numbers between L and 0.4 mg-L groups ($p > 0.05$) (Figure 3 III-B). However, osteoblast numbers

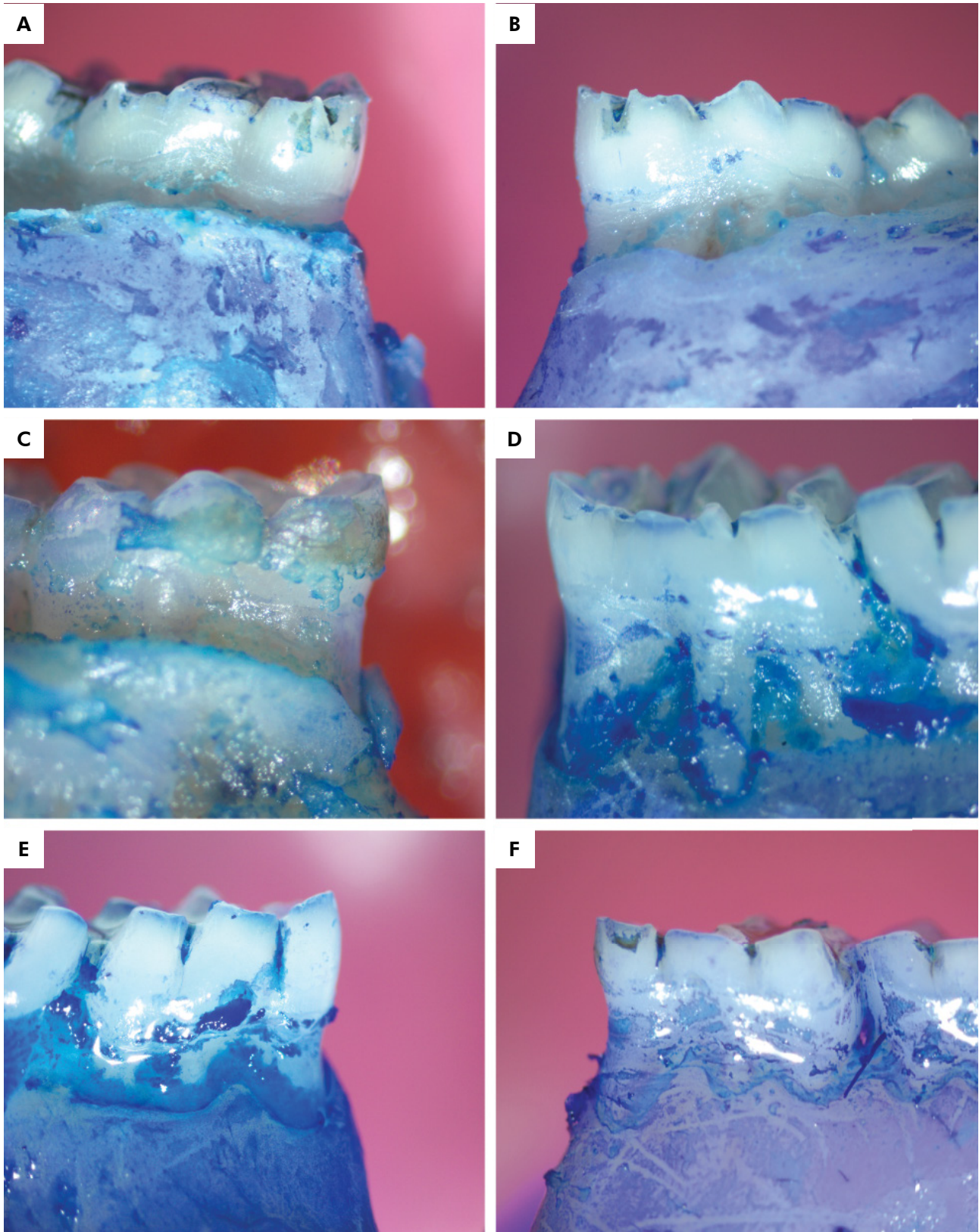
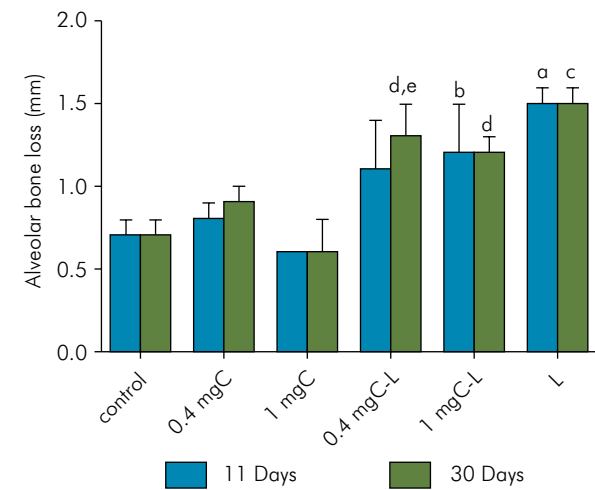


Figure 1. Representative photographs of the alveolar bone loss in the mandibular first molar tooth in the Control (A), 0.4 mgC (B), 1 mgC (C), LO (D), 0.4 mgC-L (E), and 1 mgC-L (F) groups at 30th day.

in the 1 mg-L group were higher than in the L group at 11 days ($p < 0.05$).



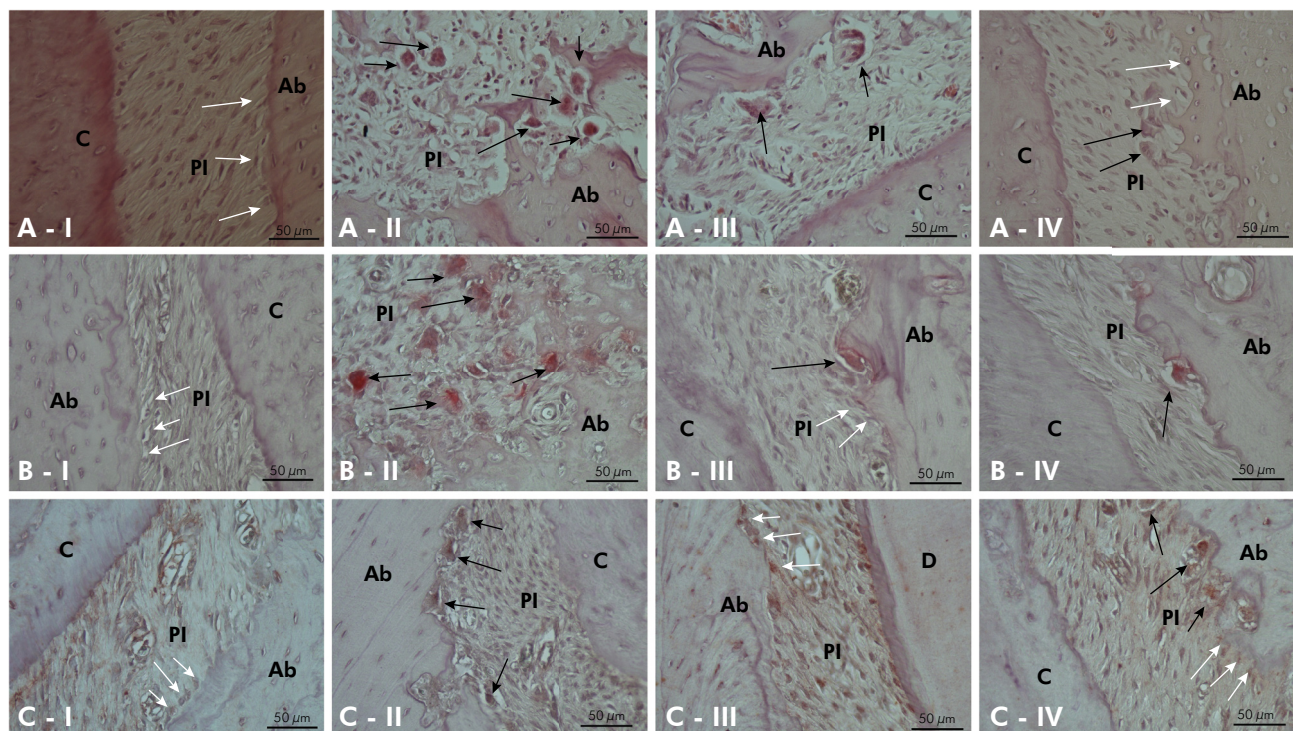
^a $p < 0.05$ vs. all controls and 0.4 mgC-L groups at 11 days;
^b $p < 0.05$ vs. C and 1 mgC groups at 11 days, $p < 0.05$ vs. all controls at 30 days; ^d $p < 0.05$ vs. C, 1 mgC group at 30 days,
^e $p < 0.05$ vs. 0.4 mgC-L at 11 days

Figure 2. Mean alveolar bone loss of study groups.

At 30 days, the lowest osteoblast numbers were observed in the L group ($p < 0.05$). Although osteoblast numbers were higher in the 1 mgC-L group than in the L group, there were no significant differences in osteoblast numbers between the L group and the 0.4 mgC-L group at 30 days. Also, osteoblast numbers were increased in the 1mgC-L group at 30 days compared to at 11 days ($p < 0.05$). Conversely, osteoblast numbers were decreased in the L group at 30 days ($p < 0.05$).

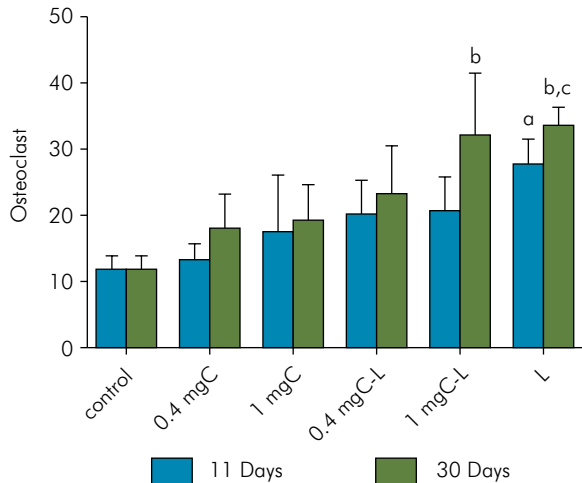
MMP-1 Immunohistochemistry

MMP-1 expression was significantly higher in the L group than in the 1 mgC, 0.4 mgC-L, or 1 mgC-L groups at 11 days ($p < 0.05$). However, there were no significant differences in MMP-1 expression between 0.4 mgC-L and 1 mgC-L groups at 11 days ($p > 0.05$). In addition, all groups expressed similar levels of MMP-1 at 30 days with no significant differences between them ($p > 0.05$) (Figure 3 I-IV C).



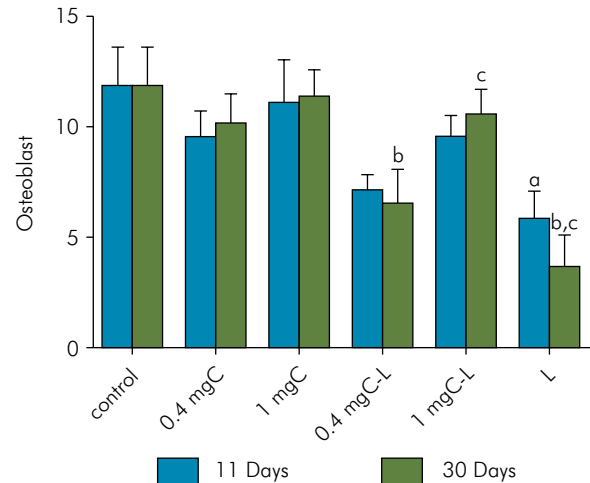
I: Non-ligated control group; II: Ligated group (30 days); III: ligated and 0.4 mg/kg colchicine administered group (30 days); IV: Ligated and 1mg/kg colchicine administered group (30 days); Ab: alveol bone; c: cement; D: dentin; PI: periodontal ligament. Black arrows show osteoclast cells and white arrows show osteoblast cells.

Figure 3. Representative samples of TRAP, hematoxylin and MMP-1-stained periodontium from Wistar rats. A: TRAP staining (400x magnification), B: Hematoxylin staining (400x magnification) and C: MMP-1 staining (400x magnification).



^ap < 0.05 vs. all controls at 11 days, ^bp < 0.05 vs. all controls at 30 days, ^cp < 0.05 vs. L group at 11 days

Figure 4. TRAP + osteoclast numbers in experimental groups and controls (mean ± SD).



^ap < 0.05 vs. all controls and 1 mgC-L at 11 days; ^bp < 0.05 vs. the other groups at 30 days; ^cp < 0.05 vs. 11 days

Figure 5. Mean osteoblast numbers in the study groups.

Discussion

This study is the first to evaluate the effect of colchicine on experimental periodontitis and alveolar bone tissue. We have demonstrated that systemically-administered colchicine does not increase alveolar bone loss at either dose and after either term in healthy periodontium. In addition, we found that colchicine tends to decrease alveolar bone loss by increasing osteoblastic activity and decreasing osteoclastic activity in ligature-induced periodontitis.

The ligature method has been extensively used for induction of experimental periodontitis.^{29,30} In this method, ligation triggers an inflammatory reaction caused by an accumulation of bacterial plaque around the first molar tooth.²⁶ In the present study, ligature placement on the first molar tooth caused a significant amount of bone loss at day 11 and 30. The eleven day and 30 day time periods are considered to be suitable time periods to observe early and late progress of the disease and treatment, relatively.^{24,25,26,27} However, for every animal model of a human disease, there are inherent limitations. Molars in rats are similar in anatomic configuration and structure to human molars, but the experimental model used is that the induced periodontitis follows an acute course, during which tissue trauma and adjacent microbial

accumulation accelerate the destructive process. Such pathways of acute inflammation are likely to differ from those involved in chronic periodontitis.

Colchicine is currently being used confidently in several diseases due to its anti-inflammatory effect.³¹⁻³² In a recent experimental study which investigated the effects of colchicine, a microtubule-disrupting agent, on skeletal muscle ischemic injury in rats, the authors suggested that 1mg/kg colchicine significantly decreased levels of malondialdehyde, TNF- α , and IL-1 β and increased superoxide dismutase in ischemic tissues.³³ In addition, at higher doses colchicine exerts various other inflammatory effects including suppression of phospholipase A2 activation, lysosomal enzyme release, and phagocytosis.³⁴⁻³⁵ However, in our study, inflammatory status was evaluated histologically and MMP-1 expression. Inflammatory cell infiltration was increased in all ligated groups due to the presence of ligatures. However, colchicine administration, especially at a dose of 0.4mg/kg, decreased ICI activity at 11 days but this effect was not seen at 30 days.

MMP-1 and -8 belong to the collagenase group of the MMP family, and it has been reported that both MMP-1 and -8, the major interstitial collagenases, degrade the extracellular matrix in periodontal disease and are mainly secreted by neutrophils.³⁶ In addition, MMP-1 may preferentially affect the

initiation of collagen destruction compared to MMP-8. Further, several reports have shown that MMP levels are higher in periodontitis, compared to gingivitis and healthy individuals.³⁷⁻³⁸ In our study, the highest expression of MMP-1 was observed in the ligature only group and the expression decreased in groups with systemically-administered colchicine. Furthermore, there was no significant effect on healthy periodontium at the evaluated doses. However, Nahm et al. found contradictory effects, reporting that colchicine-treated periodontal ligament fibroblasts significantly increased the expression of MMP-1 in a time-dependent manner compared with the controls and also showed a time-dependent increase in TIMP-1 and TGF-beta1 expression.³⁹

Several clinical studies have been conducted to investigate the association between bone metabolism and colchicine, but contradictory results have been found. Suyani et al. investigated the association between FMF and osteoporosis in adult patients who were taking regular colchicine.¹⁹ They found that total femur T scores were significantly lower in FMF patients compared to healthy controls. They also suggested that subclinical inflammation may be associated with decreased bone mineral content in these patients. In contrast, Siverekli et al.⁴⁰ suggested that no significant difference was found between FMF and healthy controls regarding bone density. In our study, osteoclast numbers were higher in the ligature only group than any others during the study period. Colchicine treatment reduced osteoclast number to control levels by 11 days, and did not cause any alveolar bone loss in the periodontium. Similarly, the results of our previous clinical study suggested that patients with FMF, who regularly used colchicine, did not manifest higher attachment loss compared to age- and sex-matched systemically-healthy controls.⁴¹

In an earlier study made by Arai et al., they reported that colchicine treatment at a dose 1mg/kg intravenously revealed high levels of type I collagen mRNA expression in osteoblasts of the mineralized ectopic surface during the bone period and formed trabecular bone-like ectopic calcified tissue.⁴² Furthermore, in that study osteocalcin showed no specific signals throughout the experiments but osteopontin mRNA was expressed especially in the initial phase of ectopic bone resorption. However, Salai et al. reported that colchicine is an *in vitro* inhibitor of proliferation of osteoblasts, causing a marked decrease in tissue mineralization.⁴³ Also, they suggested that colchicine at low concentrations, of up to 3 ng/mL, has the capacity to selectively inhibit mineralization by bone-like cell in culture, without affecting osteoblast cell proliferation. Conversely, we found that osteoblastic activity was less frequently seen in the ligature-only group and showed higher activity and numbers in colchicine groups, especially at a dose of 1 mg.

There are certain limitations to the present study. As the exact mechanism underlying the anti-inflammatory effect of colchicine in periodontitis is not clear, we evaluated limited parameters in the present study, only evaluating MMP-1 levels and inflammatory cell counts in histological sections as markers of inflammation. Changes in the expression of bone-specific markers such as RANKL and osteoprotegerin must be investigated.

In conclusion, within the inherent limitations of this animal study, we found that colchicine exerted an anti-inflammatory effect on periodontitis progression in ligature-induced periodontitis without affecting healthy periodontium. However, further studies are warranted to investigate the long-term efficacy and mode of action of colchicine on alveolar bone loss and inflammation in periodontitis because of its extensive use in several inflammatory diseases.

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