

Histological comparison of effectiveness of low doses of doxycycline and atorvastatin on gingival inflammation and alveolar bone loss in experimental model of periodontitis in rats

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Objectives The purpose of this study was to evaluate the effect of low dose doxycycline and atorvastatin on gingival inflammation and alveolar bone loss in an experimental model of periodontitis in rats.

Methods Forty male Sprague Dawley rats were divided into four study groups as follows: (I) experimental periodontitis control, (II) rats with periodontitis treated with low dose atorvastatin (10 mg/kg), (III) rats with periodontitis treated with low dose doxycycline (6 mg/kg) and rats with periodontitis treated with both doxycycline and atorvastatin. Periodontitis was induced by ligature placement around the upper left second molar for seven days. The periodontitis group received saline, periodontitis/doxycycline group received doxycycline by oral gavage, periodontitis/atorvastatin group received atorvastatin by oral gavage and doxycycline/atorvastatin group received both drugs simultaneously (6 and 10 mg/kg, respectively) for 21 days after ligature placement. Then, the rats were sacrificed and their maxillae were removed, defleshed, and prepared for histopathological examination. Data were analyzed statistically by the Kruskal-Wallis test and Mann-Whitney U test at 5% level of significance and presented as frequency.

Results Using a combination of doxycycline and atorvastatin caused a significant decrease in gingival inflammation and alveolar bone loss (16.5%) and collagen degradation (13%) when compared to the control group (36.10% and 36.95%, respectively; $P < 0.001$).

Conclusion Low dose atorvastatin and low dose doxycycline synergically prevented alveolar bone loss and collagen degradation in ligature-induced periodontitis in rats.

Keywords periodontitis, doxycycline, atorvastatin calcium, alveolar bone loss, collagen

Introduction

Periodontitis is a common clinical disorder of the periodontium in adults¹ characterized by the loss of connective tissue (CT) and alveolar bone loss around the teeth.^{2,3} It is also characterized by an acute immune response causing the production of cytokines, prostaglandins and matrix metalloproteinase as well as immune-inflammatory process regulators. Periodontal disease is one of the oral problems that has an extensive effect on the human population, and is one of the major causes of adult tooth loss.⁴

Oral bacteria and their products (e.g. lipopolysaccharides and proteases) are responsible for the initiation of periodontitis. Conversely, the progression of periodontitis depends on the host responses to bacterial pathogens. It has been observed that the invading bacteria trigger the release of cytokines such as interleukin-1 and tumor necrosis factor- α .

Antibiotics have been shown to be helpful in periodontal treatment.⁵ Tetracyclines including doxycycline have long been recognized as adjunctive antibiotics for periodontal treatment because of their effectiveness on Gram-negative anaerobic periodontal pathogens, such as *Aggregatibacter actinomycetem-comitans*, which is commonly found in the subgingival plaque.

Although initially it is attributed to their antimicrobial properties,⁶ the clinical efficacy of tetracyclines in periodontitis has been recently suggested to be attributed to their intrinsic anti-inflammatory activity.⁷ It has been approved by the US Food and Drug Administration and other national

regulatory agencies in Canada and Europe that the novel “low dose” or non-antibacterial formulations of tetracyclines, such as low dose doxycycline (LDD) can be used as an adjunctive treatment for periodontal disease. Several studies have shown that LDD suppresses host-derived matrix metalloproteinases in periodontal lesions and thereby inhibits the pathological degradation of various collagens, including types I, III, and IV; while it preserves other constituents of the periodontal tissues (fibronectin, proteoglycan ground substance, elastin, and basement membrane) and inhibits bone resorption.⁸⁻¹¹

Atorvastatin is a member of the statin class of inhibitors. Through the inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, statins have revolutionized the treatment of hypercholesterolemia. The beneficial effects of HMGCoA reductase inhibitors are attributed to their ability to reduce endogenous cholesterol synthesis.¹²

Atorvastatin is also used to inhibit metalloproteinase,¹³ osteoclastogenesis and bone destruction, as well as the expression of the receptor activator of nuclear factor- κ B (NF- κ B) ligand.^{14,15} It is yet unknown whether atorvastatin would be beneficial in a complex tissue such as the periodontium. In addition to bleeding, statins are responsible for a variety of biochemical alterations, including reduced accumulation of esterified cholesterol in macrophages, an increase in endothelial nitric oxide synthase, reduction of inflammatory processes, and increased stability of atherosclerotic plaques. Statin therapy is essential for primary and secondary prevention of

coronary artery disease.¹ Recent experimental and clinical evidence suggests that the beneficial effects of statins may extend beyond their cholesterol-lowering effects, to include the so-called pleiotropic effects.¹⁶ Since the first report by Mundy et al,¹⁷ in 1999, several statins have shown anabolic effects on bone metabolism.¹⁸

Statins stimulate osteoblastic differentiation by increasing the gene expression of bone morphogenetic protein 2 (BMP-2).¹⁹ They may also increase bone formation by inhibiting osteoblast apoptosis.¹⁸ With constant remodeling of bone, which includes bone formation and resorption, it is contributed the inhibition of osteoclast activity to the bone preservation. It has been reported that statins decrease the osteoclastic activity through the RANK-reactive oxygen species pathway.^{20,21}

The aim of the present study was to assess the combined effects of low dose atorvastatin and low dose doxycycline for treatment of periodontal disease by assessing gingival inflammation, alveolar bone loss and collagen degradation in rats with induced periodontitis.

Methods

In this animal study, 40 adult male Sprague-Dawley rats with an initial mean weight of 250 to 300 g were used. Ten rats were housed in each cage and maintained under a 12-hour light/dark cycle at a temperature of 22°C and relative humidity of 50% with access to standard rat chow pellets (Behparvar Company, Shiraz, Iran). The nutritional composition of the pellets consisted of 88% dry matter, 14% crude protein, 10% crude ash, 11% crude cellulose, 1.3% to 2.0% calcium, 1% phosphorus, 1% NaCl, and 2.6 kcal/g and ad libitum water. The Ethics Committee on Animal Experimentation of Shiraz University of Medical Sciences, Shiraz, Iran, approved the experimental protocol of the present study.

Experimental induction of periodontitis

For induction of periodontitis, general anesthesia was induced by intramuscular injection of a combination of 0.1 mL ketamine hydrochloride (50 mg/mL) and 0.05 mL xylazine hydrochloride (2 g/100 mL) for each 100 g of body weight. Sterile 5–0 cotton thread ligatures were placed subgingivally around both maxillary second molars. The rats were randomly divided into four groups of 10 rats each. In group 1, the rats were fed powdered standard chow, and periodontitis was induced.¹ In group 2 (periodontitis + low dose atorvastatin) the rats were fed powdered standard chow, periodontitis was induced and they received low dose atorvastatin (10 mg/kg).²² In group 3 (periodontitis + low dose doxycycline), the rats were fed powdered standard chow, periodontitis was induced and they received low dose doxycycline orally gavaged at 6 mg/kg dose. In group 4 (periodontitis + low dose atorvastatin + low dose doxycycline), the rats were fed powdered standard chow, periodontitis was induced and they received low dose atorvastatin and low dose doxycycline (10 and 6 mg/kg, respectively). The dose of doxycycline in rats was calculated according to the method described by Ramamurthy et al.²³ Low dose doxycycline and low dose atorvastatin were administered daily for 21 days, and after that, the rats were sacrificed.

Histological Examination

Histological evaluations were performed on the maxillary right molars. The maxillary specimens were fixed in 10%

formalin for 24 hours at room temperature and decalcified in 0.1 M ethylenediaminetetraacetic acid solution at 42°C for 10 days. Decalcification was performed as described by Cho et al,²⁴ with slight modification. Then, the specimens were dehydrated using graded alcohol series, cleared in methyl benzoate, and embedded in paraffin. The paraffin blocks were serially cut in mesiodistal direction along the long axis of the teeth to provide 6 µm-thick longitudinal sections. Care was taken to obtain histological sections in which the first and second molars, the interproximal alveolar bone crest, and the coronal and root pulp chambers were evident. Sections representative of each specimen were selected and stained with modified Mallory's connective tissue staining method.²⁵ Serial sections containing the first and second molars, in which the coronal and root pulp, cemento-enamel junction at the mesial side of the second molar, interproximal alveolar bone, and connective fiber attachment were clearly visible, were selected for histometric analysis.

Gingival inflammation and alveolar bone loss in different groups were descriptively scored from 0 to 3:

Score 0: None or small amount of inflammatory cell infiltration limited to marginal gingiva. Alveolar bone and cementum were intact.

Score 1: Moderate infiltration of inflammatory cells (inflammation of the entire gingiva) with minimal alveolar bone loss and cementum loss.

Score 2: Severe infiltration of inflammatory cells (inflammation of the entire gingiva and periodontal ligament) with moderate alveolar bone loss and partial destruction of cementum.

Score 3: Severe infiltration of inflammatory cells, complete destruction of alveolar bone and severe destruction of cementum.

Collagen degradation in different groups was evaluated descriptively and scored as: normal = 0, mild = 1, moderate = 2 or severe = 3.

Statistical analyses

Data were analyzed by SPSS version 19.0 (SPSS Inc., IL, USA) and presented as mean ± standard deviation. The non-parametric Kruskal-Wallis test was applied to determine whether there was any significant difference between the groups. The Mann-Whitney U test was performed to detect the differences in the frequency of each marker between the experimental groups. Differences were interpreted as significant at $P < 0.05$.

Results

Combined treatment decreased gingival inflammation and alveolar bone loss

The results revealed that gingival inflammation and alveolar bone loss significantly decreased after 21 days of treatment with 10 mg/kg/day atorvastatin plus 6 mg/kg/day doxycycline (16.50% versus 36.10% in periodontitis group).

Atorvastatin and doxycycline alone had a protective effect on alveolar bone of rats with periodontitis (16.79% and 21.18%, respectively compared to the control group); however, there was no significant difference between the treatment groups (low dose doxycycline, low dose atorvastatin and both) ($P > 0.05$).

Table 1. Effect of low dose doxycycline, low dose atorvastatin and combination of both on alveolar bone loss in rats with periodontitis

Group	Normal	Mild	Moderate	Severe	Mean rank
Control ¹ (n = 10)	0	0	30	70	36.10
LDA ² (n = 10)	0	41.7	41.7	16.7	21.18*
LDD ³ (n = 10)	0	58.3	41.7	0	16.79*
LDA + LDD ⁴ (n = 10)	0	60	40	0	16.50*

Data are presented as frequency of 10 or 12 rats in each group by Kruskal-Wallis test and Mann-Whitney U test. 1, Periodontitis; 2, Periodontitis + low dose atorvastatin; 3, Periodontitis + low dose doxycycline; 4, Periodontitis + low dose atorvastatin + low dose doxycycline; *, $P < 0.001$ compared with periodontitis control group.

Table 2. Effect of low dose doxycycline, low dose atorvastatin and combination of both on collagen destruction in rats with periodontitis

Group	Normal	Mild	Moderate	Severe	Mean rank
Control ¹ (n = 10)	0	10	70	20	36.95
LDA ² (n = 10)	50	41.7	8.3	0	22.21*
LDD ³ (n = 10)	66.7	33.3	0	0	18.67*
LDA + LDD ⁴ (n = 10)	90	10	0	0	13.00*

Data are presented as frequency of 10 or 12 rats for each group by Kruskal-Wallis test and Mann-Whitney test. 1, Periodontitis; 2, Periodontitis + low dose atorvastatin; 3, Periodontitis + low dose doxycycline; 4, Periodontitis + low dose atorvastatin + low dose doxycycline; *, $P < 0.001$.

Tables 1 and 2 shows the results of histopathological examination of rats with periodontitis (control group) with intense alveolar bone loss, compared to treatment groups.

Combined treatment decreased collagen degradation

Rats with periodontitis (36.95%) showed significantly higher collagen destruction compared with low dose atorvastatin (22.21%), low dose doxycycline (18.67%) or combination of both (13.00%) ($P < 0.001$). In other words, doxycycline and atorvastatin alone were effective but combined administration of both was the most effective in reducing collagen degradation when compared with periodontitis control group ($P = 0.000$), low dose doxycycline ($P = 0.000$) and low dose atorvastatin group ($P = 0.001$). Figure 1 shows macroscopic images of alveolar bone loss and collagen degradation in low dose doxycycline, low dose atorvastatin and combined group compared to the periodontitis control group.

Discussion

Results of the present study showed that combination of low dose doxycycline (6 mg/kg) and atorvastatin (10 mg/kg) can cause protection against the destruction of alveolar bone and collagen in rats with induced periodontitis.

It was also found that atorvastatin and doxycycline alone were capable of inhibiting inflammation and bone loss associated

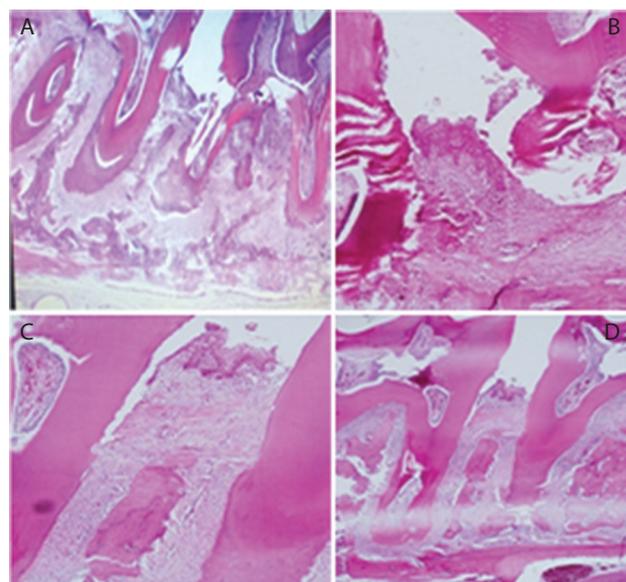


Fig 1. Clinical characteristics of teeth and periodontal tissue; (A) samples of experimental periodontitis showing resorption of the alveolar bone in score 3; (B) samples from LDD group with resorption of the alveolar bone in score 2; (C) samples from LDA group with resorption of the alveolar bone in score 1; (D) samples from LDD + LDA group with resorption of the alveolar bone in score 1.

with collagen degradation, but there was no significant difference between the protective effects of the two drugs.

A significant reduction in alveolar bone resorption and collagen degradation was noted in the experimental periodontitis group after doxycycline administration.

Doxycycline is one of the most potent and cost-effective antibiotics, which has been shown to reduce collagen degradation.²⁶ Our results, similar to those of Ozdemir et al.²⁷ and Bezerra et al.¹¹ showed that low-dose (sub-antimicrobial) doxycycline prevented alveolar bone loss at the macroscopic level. Doxycycline is 14 times stronger than tetracycline and at low concentrations (2, 5–10 mg per mL) has the ability to inhibit collagenase secreted by neutrophils and *Porphyromonas gingivalis*. Another mechanism suggested for this inhibition by doxycycline is the inhibition of matrix metalloproteinase and stress oxidative.²³

Yagan et al.²⁸ consistent with our study indicated that periodontitis was induced in rats after 7 days of placement of cotton ligatures around teeth. Moreover, they reported that low-dose doxycycline (6 mg/kg) for 21 days by oral gavage prevented periodontal tissue breakdown by inhibiting local and systemic oxidative stress. However, unlike our study, they investigated the effect of doxycycline on the oxidant and anti-oxidant levels in sera and also histologically.

An increase in oxidants and free radicals produced during inflammation is one of the mechanisms of tissue pathogenesis. It seems that increased serum levels of oxidants and decreased antioxidant enzymes promote inflammation and alveolar bone loss by activation of pro-inflammatory cytokines such as IL-6, interleukin-1 and TNF- α and subsequently activated RANKL protease enzymes (osteoclast differentiation stimulator).

Our results consistent with those of other studies²⁹⁻³¹ demonstrated the effectiveness of the oral administration of atorvastatin to prevent alveolar bone resorption in rats with experimental periodontitis.

Several authors have reported the successful use of atorvastatin for periodontitis, by reducing tooth loss in patients with chronic periodontitis,²⁹ minimizing the signs/symptoms of inflammation.³⁰

Fentoğlu et al³² reported that atorvastatin decreased pro-inflammatory cytokines in addition to lipid factors in periodontal and hyperlipidemia patients. Also, de Araújo Júnior et al.³³ showed that 10 mg/kg of atorvastatin can reduce bone loss, pro-inflammatory cytokines, oxidative stress, and expression of extracellular matrix proteins, as well as RANK/RANKL while it increases osteoprotegerin in male Wistar albino rats with periodontitis.

Conclusion

Our result indicated that atorvastatin and doxycycline can reduce alveolar bone loss in ligature-induced periodontitis.

Combination of low dose atorvastatin and doxycycline may exert beneficial effects on metabolic and inflammatory conditions in periodontitis. It seems that both drugs play multifunctional roles in inhibition of experimental periodontitis in rats. Hence, synergic use of atorvastatin and doxycycline is more efficient. However, further studies are required to find out more about the possible side effects.

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Conflict of Interest

“None Declared.” ■

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