Journal of
PERIODONTAL RESEARCH

J Periodont Res 2014; 49: 45–54

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/jre.12077

Low-dose combination of alendronate and atorvastatin reduces ligature-induced alveolar bone loss in rats

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Goes P, Melo IM, Silva LMCM, Benevides NMB, Alencar NMN, Ribeiro RA, Lima V. Low-dose combination of alendronate and atorvastatin reduces ligature-induced alveolar bone loss in rats. J Periodont Res 2014; 49: 45–54. © 2013 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

Background and Objective: Atorvastatin (ATV) has bone anabolic properties, and alendronate (ALD) is an important antiresorptive drug. This study aimed to evaluate the effects of the combination of ALD and ATV on ligature-induced alveolar bone loss in rats.

Material and Methods: Periodontitis was induced by ligature in 78 Wistar rats. Groups of six rats prophylactically received 0.9% saline (SAL), ALD (0.01 or 0.25 mg/kg subcutaneously) or ATV (0.3 or 27 mg/kg by gavage). Then, groups of six rats received the combination of ALD+ATV (0.25 mg/kg + 27 mg/kg, 0.01 mg/kg + 0.3 mg/kg, 0.25 mg/kg + 0.3 mg/kg or 0.01 mg/kg + 27 mg/kg) prophylactically. An extra group of six rats received therapeutic SAL or a lower-dose combination of ALD+ATV (0.01 mg/kg + 0.3 mg/kg, respectively) therapeutically. Three extra groups of six rats each received SAL or a lower-dose combination of ALD+ATV (0.01 mg/kg + 0.3 mg/kg, respectively) prophylactically or therapeutically for histometric and immunohistochemical analyses. The rats were killed on day 11 after ligature placement, and the maxillae were removed and processed for macroscopic, histomorphometric and TRAP immunohistochemical analyses. Gingival samples were collected to evaluate myeloperoxidase (MPO) activity. Blood samples were collected to measure serum bone-specific alkaline phosphatase (BALP) and transaminase levels and for hematological studies. Rats were weighed daily.

Results: All combined therapies prevented alveolar bone loss when compared with SAL or low doses of monotherapy (ALD or ATV) ($p < 0.05$). The lower-dose combination of ALD+ATV (0.01 mg/kg + 0.3 mg/kg, respectively), administered either prophylactically (39.0%) or therapeutically (53.5%), prevented alveolar bone loss. Decreases in bone and cementum resorption, in leukocyte infiltration and in immunostaining for TRAP and MPO activity corroborated the morphometric findings. The lower-dose combination of ALD+ATV (0.01 mg/kg + 0.3 mg/kg, respectively) prevented BALP reduction ($p < 0.05$) and did not alter the level of serum transaminases. Moreover, the lower-dose combination of ALD+ATV (0.01 mg/kg + 0.3 mg/kg, respectively) also reduced neutrophilia and lymphomonocytosis and did not cause weight loss when compared with administration of SAL.

Conclusion: The lower-dose combination of ALD+ATV (0.01 mg/kg + 0.3 mg/kg, respectively) demonstrated a protective effect on alveolar bone loss.

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Key words: alendronate; alveolar bone loss; atorvastatin; inflammation

Accepted for publication February 12, 2013

Periodontitis is a multifactorial disease that involves biofilms and an inflammatory response, which leads to the production of cytokines, eicosanoids, MMPs, and other inflammatory factors, resulting in bone loss (1).

The bone remodeling cycle is controlled by a variety of mechanisms. The discovery of osteoprotegerin/RANK/RANKL system provide insight into a major component of the remodeling cycle. The expression of RANKL on the surface of osteoblasts is up-regulated in response to a variety of proresorptive signals, including pro-inflammatory cytokines [tumor necrosis factor-alpha, interleukin (IL)-1β and IL-6], which are present on the periodontal site and will stimulate osteoclastogenesis (2).

Pharmacological approaches to prevent or treat bone disease have been developed bearing in mind the importance of the host response in this process. Alendronate (ALD) and atorvastatin (ATV) stand out among a variety of drugs for the treatment of bone disease. ALD, bisphosphonate (BP), has been used as a therapeutic agent for the management of osteoporosis, Paget's disease and bone metastasis (3). The antiresorptive effect of BPs has been attributed to their inhibitory effect on osteoclasts. BPs decrease osteoclast maturation, which promotes apoptosis of mature osteoclasts by inhibiting the effects of farnesyl diphosphate synthase, a key enzyme in the mevalonate pathway (4). The antiresorptive effect of ALD has been confirmed, in preclinical (5) and clinical assays (6), on both systemic and local periodontitis.

ATV, a statin, is widely used for lowering cholesterol levels and it has been reported that statins have pleiotropic effects, such as antioxidant properties, inhibition of inflammatory responses and bone anabolic effects (7), and the beneficial effects of ATV on alveolar bone have been reported in studies in animals (8) as well as in humans (9). In addition, statins directly affect osteoclasts through mechanisms analogous to those of BPs. Both statins and BPs exert their effects through the mevalonate pathway (10). Therefore, considering that both ALD and ATV act through the mevalonate pathway and have been shown to have antiresorptive, anti-inflammatory and bone anabolic actions, this study aimed to evaluate, for the first time, the prophylatic and therapeutic effects of the combination of ALD and ATV on ligature-induced alveolar bone loss in rats.

Material and methods

Animal selection

Seventy-eight male Wistar rats (*Rattus norvegicus*) from our own facilities, weighing 180–220 g, were used. The rats were acclimatized for 1 week before the assay and housed under normal conditions, with laboratory chow and water available *ad libitum*. Experimental protocols were executed following the ethical principles for laboratory animal use and were approved by the Institutional Ethical Committee of Animal Research (protocol no. 101/2009).

Ligature-induced alveolar bone loss

The rats were divided into groups, with six rats in each. Alveolar bone loss was induced using the periodontal model, previously described (11), by placing a nylon ligature around the cervix of the second left upper molar of rats anesthetized with chloral hydrate (Vetec Química Fina®; Duque de Caxias, RJ, Brazil). The ligature was knotted on the buccal side. The contralateral side was used as an unligated control. Rats were observed until day 11 after ligature placement, the day of the most intense alveolar bone loss (11), and then were killed.

Experimental groups

Saline group— Six rats subjected to the periodontal model of alveolar bone loss received 2 ml/kg of 0.9% sterile saline solution (SAL) by gavage 30 min before ligature placement. The same volume of SAL was administered daily after ligature placement until day 11, when the rats were killed.

ALD groups— Rats were divided into two groups of six rats each. Alendronate (ALD) (Fosamax®; Merck, São Paulo, SP, Brazil) was dissolved in 0.9% SAL and administered, subcutaneously, at doses of either 0.01 or 0.25 mg/kg (12), 30 min before ligature placement and daily thereafter until day 11, when the rats were killed.

ATV groups— Rats were divided in two groups of six rats each. Each group received ATV (Lipitor®; Pfizer, São Paulo, SP, Brazil), by gavage, at doses of either 0.3 or 27 mg/kg (9), 30 min before ligature placement and daily thereafter until day 11, when the rats were killed.

ALD +ATV groups: prophylactic regimen— Rats were divided into four groups of six rats each. They received ALD+ATV 30 min before ligature placement and daily thereafter until day 11, when they were killed. The following combinations were used: low doses (ALD 0.01 mg/kg + ATV 0.3 mg/kg); high/low or low/high doses (ALD 0.25 mg/kg + ATV 0.3 mg/kg and ALD 0.01 mg/kg + ATV 27 mg/kg, respectively); or high doses (ALD 0.25 mg/kg + ATV 27 mg/kg). ATV was administered by gavage, and ALD was administered subcutaneously.

ALD +ATV group: therapeutic regimen— A group of six rats was subjected to ligature-induced alveolar bone loss. The rats received a lower-dose combination of ALD+ ATV (0.01 mg/kg + 0.3 mg/kg, respectively) from day 5 (13) to day 11 after ligature placement, at which point the rats were killed. ATV was administered orally, and ALD was administered subcutaneously.

Macroscopic study of bone tissue

The maxillae were removed on day 11 after ligature placement and fixed, for 24 h, in 10% formaldehyde (Reagen Produtos para Laboratórios Ltda). Morphometric analysis was performed as follows: the maxillae were separated in half, defleshed, stained with 1%

aqueous methylene blue (Vetec Quimca Fina), placed on microscope slides and photographed using a digital camera (Nikon Inc D40; Melville, NY, USA). Resorption was measured in the area including the occlusal tips of the vestibular side of the teeth to the bone border in the entire maxilla. The images were evaluated using IMAGE J® software (Software ImageJ 1.32j; National Institutes of Health, Bethesda, MD, USA) (8,11).

Histometric study

The same slides used for histologic analyses were used for histometric studies. Slide inclusion criteria were the presence of dental root, epithelial tissue and interproximal bone in the same section. Images of these sections were captured at 40 × magnification. After excluding the first and the last sections, four subsequent sections of each tooth were selected for histometric analysis (14). A trained observer used IMAGE J® software to obtain three linear measurements from the alveolar bone crest to the cemento–enamel junction at the mesial root of the second upper molar. The distance from the alveolar bone crest to the cemento–enamel junction was calculated as the mean of three measurements.

Histologic analysis of alveolar bone

Three groups, of six rats each, were subjected to periodontitis and received SAL or the lower-dose combination of ALD+ATV (0.01 mg/kg + 0.3 mg/kg), prophylactically or therapeutically. On day 11 after ligature placement, the rats were killed and the maxillae were excised from the rats. The specimens were fixed in 10% formaldehyde and demineralized in 7% formic acid (Merck, Jacarepaguá, RJ, Brazil) for 10 d. The specimens were then rehydrated, embedded in paraffin, sectioned in the buccal-lingual direction in the mesiodistal plane and stained using hematoxylin and eosin. Sections of 4 μm thickness were evaluated using light microscopy (40 × magnification). Inflammatory cell infiltration, osteoclast number, and alveolar bone and cementum integrity were graded by scores of 0–3 based on the intensity of the findings (11).

TRAP immunohistochemical staining

Histologic sections of rats subjected to periodontitis and that received SAL or the lower-dose combination of ALD+ATV (0.01 mg/kg + 0.3 mg/kg), either prophylactically or therapeutically, were subjected to an indirect immunoperoxidase assay using a polyclonal antibody for TRAP identification. All reactions were accompanied by a negative control performed through the omission of primary (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or secondary (Jackson Immunoresearch Laboratories, West Grove, PA, USA) antibody (15).

Myeloperoxidase activity

Myeloperoxidase (MPO) activity was evaluated in samples of gingival tissue from the second upper molar (16). Rats that received SAL or the lower-dose combination of ALD+ATV (0.01 mg/kg + 0.3 mg/kg, respectively) in prophylactic and therapeutic regimens had a sample of the challenged gingiva removed on day 11 after ligature placement for analysis of MPO activity. Gingivae of the contralateral hemimaxillae of rats that received SAL were used as the control. The specimens were stored at −80°C until required for assay. Gingivae were weighed and triturated using a Polytron Ultra-Turrax in ice-cold buffer, and the homogenate was centrifuged (3000 ***g*** at 4°C for 15 min). Supernatant was collected for MPO activity, which was determined by measuring the change of absorbance at 450 nm.

Systemic parameters

Serum levels of bone-specific alkaline phosphatase— Blood samples were collected from the orbital plexus of anesthetized rats that received SAL or the lower-dose combination of ALD+ATV (0.01 mg/kg + 0.3 mg/kg, respectively) in prophylactic and therapeutic regimens before the experiment and on day 11 after ligature placement. Bone-specific alkaline phosphatase (BALP), a thermosensible isoform of total alkaline phosphatase, was evaluated using a thermoactivation method. The samples were heated to 56°C for 10 min (17). Serum levels of BALP were calculated by subtracting the concentration of heated alkaline phosphatase in serum from the concentration of total alkaline phosphatase in serum. These analyses were performed according to the manufacturer's instructions (Labest, Lagoa Santa, MG, Brazil).

Serum level of transaminases (aspartate aminotransferase and alanine aminotransferase)— At baseline and on day 11 after ligature placement, blood samples were collected from the orbital plexus of anesthetized rats that received SAL or the lower-dose combination of ALD+ATV (0.01 mg/kg + 0.3 mg/kg, respectively) in prophylactic and therapeutic regimens. Liver function was evaluated through measurement of the serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). These analyses were performed following the manufacturer's instructions (Labtest).

Hematologic study— The method for analysis of white blood cell, neutrophil and mononuclear cell counts was as follows: 20 μL of blood was taken from the rat tail and added to 380 μL of Turk's solution. Total white blood cell counts were performed using a Neubauer chamber, and the differential counts were made using smears stained by a rapid Instant Prov Stain Set (Newprov Products for Laboratory, Pinhais, PR, Brazil). White blood cell counts of the groups of rats that received SAL or the lower-dose combination of ALD+ATV in prophylactic and therapeutic regimens were performed before periodontitis induction, and 6 h and 2, 7 and 11 d after ligature placement.

Corporal mass variation— The rats from groups that received SAL or the lower-dose combination of ALD+ATV (0.01 mg/kg + 0.3 mg/kg, respectively) in prophylactic and therapeutic regimens had their body mass measured

before periodontitis induction and daily thereafter until day 11 after ligature placement.

Statistical analysis

The data are presented as mean ± standard error of the mean or as median (range), where appropriate. ANOVA followed by the Bonferroni test or the Student's *t*-test were used to compare the means, and Kruskal–Wallis and Dunn tests were used to compare the medians. A p value of < 0.05 was considered to indicate significant differences. All calculations were performed using the GraphPad Prism 5 software (GraphPad, Inc., San Diego, CA, USA). All protocols and analyses were performed in a blinded manner.

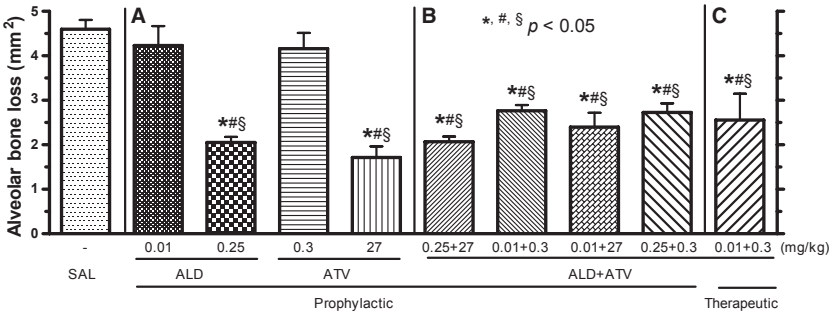

Fig. 1. Effect of alendronate (ALD) and atorvastatin (ATV) on bone tissue. The bars represent the mean±standard error of the mean of six rats per group receiving prophylactic monotherapy with high and low doses of ALD or ATV (A), the prophylactic combination of different doses of ALD+ATV (B) and the therapeutic combination of a lower doses of ALD + ATV (0.01 mg/kg + 0.3 mg/kg, respectively) (C). *Significant difference ($p < 0.05$) compared with the saline (SAL) group. #Significant difference ($p < 0.05$) compared with ALD (0.01 mg/kg). §Significant difference ($p < 0.05$) compared with ATV (0.3 mg/kg). (Differences between mean values were calculated using ANOVA followed by the Bonferroni test.)

Results

Macroscopic study of bone tissue

Ligature placement induced intense alveolar bone loss (Fig. 1), root exposure and furcation lesions (Fig. 2D), when compared with unchallenged periodontium (Fig. 2A). Monotherapy with the lowest doses of ALD and ATV did not prevent alveolar bone loss (ALS 0.01 mg/kg = 4.23 ± 0.44 mm^2 alveolar bone loss; ATV 0.3 mg/kg = 4.16 ± 0.35 mm^2 alveolar bone loss) when compared with SAL (Fig. 1A); however, prophylactic monotherapy using the highest doses of ALD and ATV significantly prevented alveolar bone loss (ALD 0.25 mg/kg = 2.1 ± 0.1 mm^2 alveolar bone loss; ATV 27 mg/kg = 1.7 ± 0.2 mm^2 alveolar bone loss) when compared with SAL (SAL=4.6 ± 0.2 mm^2 alveolar bone loss) ($p < 0.05$).

All combinations of ALD+ATV administered prophylactically significantly prevented ($p < 0.05$) alveolar bone loss – ALD 0.25 mg/kg+ ATV 27 mg/kg = 2.1 ± 0.1 mm^2 alveolar bone loss; ALD 0.01 mg/kg+ ATV 0.3 mg/kg= 2.8 ± 0.1 mm^2 alveolar bone loss; ALD 0.01 mg/kg + ATV 27 mg/kg = 2.4 ± 0.3 mm^2 alveolar bone loss; and ALD 0.25 mg/kg + ATV 0.3 mg/kg = 2.7 ± 0.2 mm^2 alveolar bone loss – compared with SAL (Fig 1B) or with low doses of monotherapy with either ALD or ATV (Fig. 1B). When the lower-dose combination of ALD+ATV was administered therapeutically, significant protection of alveolar bone (2.5 ± 0.6 mm^2 alveolar bone loss) (Fig. 2J) was also observed compared with administration of SAL or low-dose monotherapy with either ALD or ATV (Fig. 1C).

Histometric analysis of alveolar bone

The histometric study corroborated the morphometric and histological findings. It was observed that 11 d of ligature-induced alveolar bone loss caused intense alveolar bone loss when compared with the unchallenged periodontium. The lower-dose combination of ALD+ATV (0.01 mg/kg + 0.3 mg/kg, respectively), administered prophylactically or therapeutically, prevented and reduced alveolar bone loss compared with the administration of SAL ($p < 0.05$) (Table 1).

Immunohistochemistry for TRAP

Immunohistochemical staining for TRAP on the region between the first and second molars revealed intense labeling in the SAL group (Fig. 2F) compared with the unchallenged hemimaxilla (Fig. 2C). The lower-dose combination of ALD+ATV (0.01 mg/kg + 0.3 mg/kg, respectively), administered prophylactically, did not show labeling of TRAP (Fig. 2I), and the therapeutic regimen of ALD+ATV revealed a low level of immunostaining when compared with administration of SAL (Fig. 2L).

Histologic analysis of alveolar bone

In the microscopic study, it was observed that resorption of alveolar bone and of cementum were associated with an important inflammatory infiltrate ($p < 0.05$) in rats in the SAL group (Table 1; Fig. 2E) when compared with unchallenged periodontium (Table 1; Fig. 2B) ($p < 0.05$). The lower-dose combination of ALD + ATV (0.01 mg/kg + 0.3 mg/kg, respectively), administered prophylactically or therapeutically, reduced the degree of inflammatory infiltration, and preserved the periodontal ligament, root cementum and alveolar bone (Table 1; Figs. 2H and K, respectively), compared with administration of SAL ($p < 0.05$).

MPO activity

Figure 3 shows that rats subjected to 11 d of ligature presented a significant increase of MPO activity in gingival tissue (53.2 ± 12.7 MPO activity/g of tissue) when compared with unchallenged rats (0.7 ± 0.2 MPO activity/g of tissue). The lower-dose combination of ALD +ATV (0.01 mg/kg + 0.3 mg/

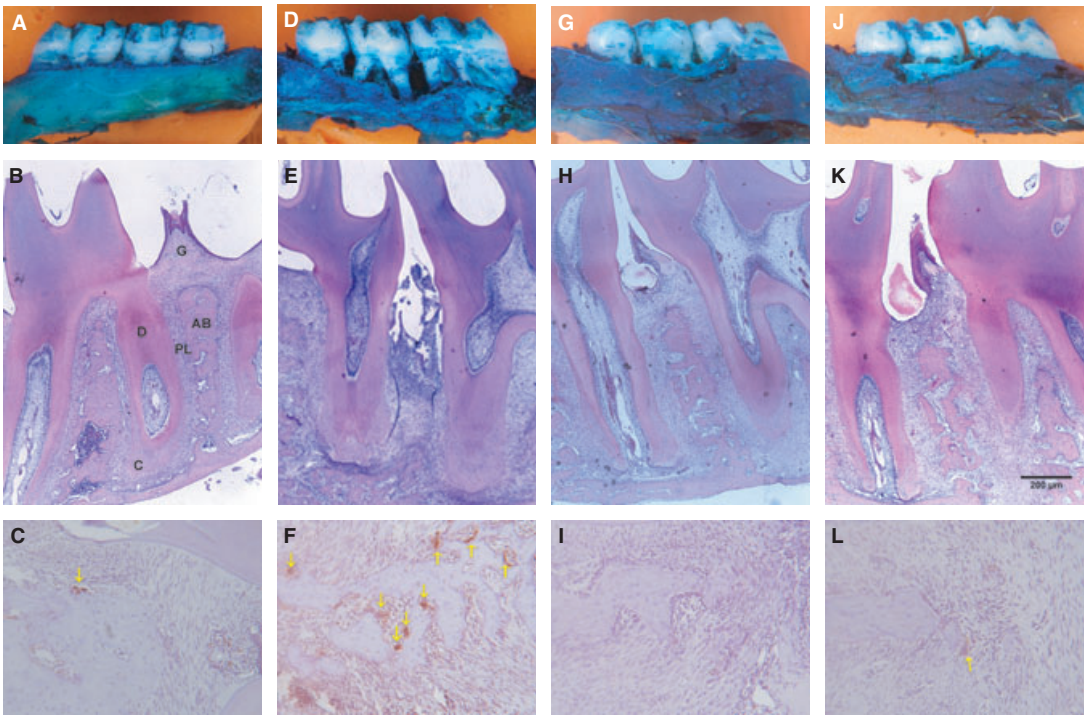

Fig. 2. Macroscopic, microscopic and immunohistochemical findings of the unchallenged periodontium (A, B and C) and of the periodontium of rats subjected to periodontitis and receiving saline (SAL) (D, E and F), prophylactic treatment (G, H and I), or therapeutic treatment (J, K and L) of combinations of lower doses of alendronate (ALD) and atorvastatin (ATV) (0.01 mg/kg + 0.3 mg/kg, respectively). The removed maxillae were dissected and photographed or were processed for hematoxylin and eosin staining or for immunostaining for TRAP. Arrows indicate TRAP immunostaining. AB, alveolar bone; C, cementum; D, dentin; G, gingiva; PL, periodontal ligament. (A, D, G, J) Macroscopic original magnification, 7 ×; (B, E, H, K) microscopic original magnification, 40 ×; and (C, F, I, L) microscopic original magnification, 250 ×.

Table 1. Histologic analysis of unchallenged hemimaxillae and of the hemimaxillae of rats subjected to periodontitis and administered saline (SAL) or the combination of lower-dose alendronate (ALD) and atorvastatin (ATV) (ALD 0.01 mg/kg + ATV 0.3 mg/kg)

Analysis	Unchallenged	SAL	ALD+ATV prophylactic	ALD+ATV therapeutic
Histologic (scores)	0 (0–0)	3 (2–3)$^{\#}$	1 (1–3)*	1 (1–2)*
Histometric (mm)	0.23 ± 0.01	0.80 ± 0.04$^{\#}$	0.62 ± 0.06*	0.50 ± 0.02*,§

Values are given as median (score range) of six rats per group or as mean ± standard error of the mean of six rats per group.
*Significant difference compared with the SAL group.
$^{\#}$Significant difference compared with unchallenged hemimaxillae.
§Significant difference compared with the hemimaxillae of rats treated with ALD+ATV prophylactically.Differences between median values were calculated using Kruskal–Wallis and Dunn tests; differences between mean values were calculated using ANOVA followed by the Bonferroni test; $p < 0.05$.

kg) prevented this increase in MPO activity after 11 d of ligature-induced alveolar bone loss, when administered prophylactically (14.2 ± 4.4 MPO activity/g of tissue) or therapeutically (10.9 ± 3.2 MPO activity/g of tissue), compared with administration of SAL ($p < 0.05$).

Serum levels of BALP

The SAL group presented a significant decrease (51.9%) in BALP serum levels on day 11 after ligature placement when compared with baseline. The lower-dose combination of ALD+ATV (0.01 mg/kg + 0.3 mg/kg, respectively), administered prophylactically or therapeutically, resulted in a significant ($p < 0.05$) increase of BALP serum levels compared with the SAL group 11 d after induction of periodontitis (Table 2).

Serum levels of transaminase (AST and ALT) activity

There was no significant difference in the serum levels of transminases in the SAL group between day 11 and baseline. The lower-dose combination of ALD+ATV (0.01 mg/kg + 0.3 mg/kg, respectively), administered prophylactically or therapeutically, did not result in any significant alteration of the AST or ALT serum levels ($p > 0.05$) (Table 2).

Hematologic study

Leukocytosis (23.4 ± 1.0 × 10^3cells/mm^3) was observed 6 h after ligature

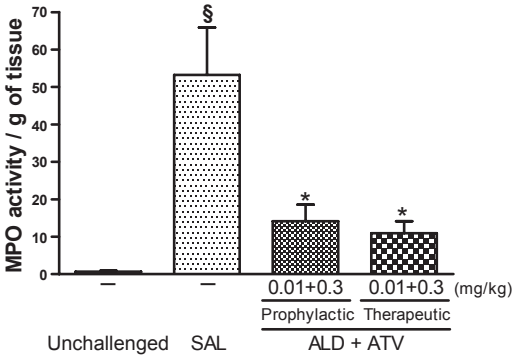

Fig. 3. Effect of prophylactic or therapeutic combinations of lower doses of alendronate (ALD) and atorvastatin (ATV) on myeloperoxidase (MPO) activity. The bars represent the mean±standard error of the mean of MPO activity/g of gingival tissue of six rats per group. *Significant difference ($p < 0.05$) compared with administration of saline (SAL). §Significant difference ($p < 0.05$) compared with the unchallenged rats. (Differences between mean values were calculated using ANOVA followed by the Bonferroni test.)

Table 2. Serum levels of bone-specific alkaline phosphatase (BALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) of rats subjected to periodontitis and receiving saline (SAL) or prophylactic or therapeutic combinations of lower-dose alendronate (ALD) and atorvastatin (ATV) (ALD 0.01 mg/kg + ATV 0.3 mg/kg)

		Groups		
	Days	SAL	ALD+ATV prophylactic	ALD+ATV therapeutic
BALP (U/L)	0	25.5 ± 3.4	24.5 ± 2.8	22.9 ± 3.3
	11	13.2 ± 1.7§	20.3 ± 2.4*	28.1 ± 3.2*
AST (U/L)	0	36.2 ± 1.7	36.2 ± 2.1	31.9 ± 1.0
	11	36.9 ± 2.7	41.6 ± 1.7	40.3 ± 3.0
ALT (U/L)	0	27.9 ± 2.5	25.1 ± 1.3	32.5 ± 3.1
	11	25.3 ± 3.2	28.6 ± 2.4	32.9 ± 2.0

Values are given as mean ± standard error of the mean of six rats per group.
§Significant difference compared with the respective baseline value.
*Significant difference compared with the results obtained in rats subjected to periodontitis for 11 d and administered SAL.
Significance was calculated using two-way ANOVA followed by the Bonferroni test and the Student's *t*-test ($p < 0.05$).

placement (Fig. 4A) compared with rats not subjected to ligature-induced alveolar bone loss ($11.9 \pm 0.8 \times 10^3$ cells/mm^3). This leukocytosis was marked by neutrophilia ($6.2 \pm 0.8 \times 10^3$ cells/mm^3) (Fig. 4B). On day 2 after ligature placement, cell counts were similar to basal cell counts ($13.1 \pm 0.8 \times 10^3$ cells/mm^3) ($p > 0.05$). Leukocytosis was again observed on days 7 ($16.2 \pm 0.8 \times 10^3$ cells/mm^3) and 11 ($24.6 \pm 1.4 \times 10^3$ cells/mm^3) after ligature placement, with mononuclear cells predominating (Fig. 4C). The lower-dose combination of ALD+ATV (0.01 mg/kg + 0.3 mg/kg, respectively), administered prophylactically ($1.7 \pm 0.3 \times 10^3$ cells/mm^3) or therapeutically ($1.5 \pm 0.2 \times 10^3$ cells/mm^3) prevented neutrophilia at 6 h compared with the SAL group ($p < 0.05$) (Fig. 4B). The lower-dose combination of ALD+ATV (0.01 mg/kg + 0.3 mg/kg, respectively) in both regimens (prophylactic = $15.7 \pm 2.2 \times 10^3$ cells/mm^3; therapeutic = $14.9 \pm 1.5 \times 10^3$ cells/mm^3) also reduced ($p < 0.05$) the mononuclear cell counts on day 11 after ligature placement compared with the SAL group ($23.1 \pm 1.2 \times 10^3$ cells/mm^3) (Fig. 4C).

Corporal mass variation

The induction of periodontitis caused body weight loss starting on day 1, and the weight loss continued until day 3 ($p < 0.05$). Then, the rats gained weight following a kinetic curve similar to that observed for unchallenged rats. The lower-dose combination of ALD+ATV (0.01 mg/kg + 0.3 mg/kg, respectively), administered prophylactically or therapeutically, did not prevent the initial body weight loss. However, the prophylactic combination initiated a greater weight gain of the rats from day 8 after ligature placement until the last day of the experiment ($p < 0.05$) compared with the SAL group (data not shown).

Discussion

In this study, it was observed that the placement of a ligature caused intense alveolar bone loss. Measurements in the proximal area demonstrated augmentation of the distance between the alveolar bone crest and the cemento–enamel junction. Moreover, intense immunostaining for TRAP was also observed. The histopathology showed intense alveolar bone loss and cementum loss and a marked influx of cells into the periodontium, which was accompanied by an increase of MPO activity in the gingival tissue. A decrease in BALP serum levels was also observed. These findings are in agreement with those of other authors (14,18–20). Periodontitis did not cause any systemic alterations in the levels of liver enzymes. Leukocytosis, with significant neutrophilia 6 h after ligature placement, and lymphomonocytosis on day 11, was also observed. An initial loss of weight was observed, which was probably caused by the trauma of the ligature placement. Altogether, this alveolar bone loss reproduced the changes previously reported in rats, of severe local inflammatory reactions and alveolar bone loss coupled with leukogram alteration (11,18).

Considering the role of inflammation on alveolar bone loss, we decided to evaluate the effects of ALD and ATV in ligature-induced alveolar bone loss in rats, a well-established model (9,11,16,18–20). These drugs were tested because BPs have been

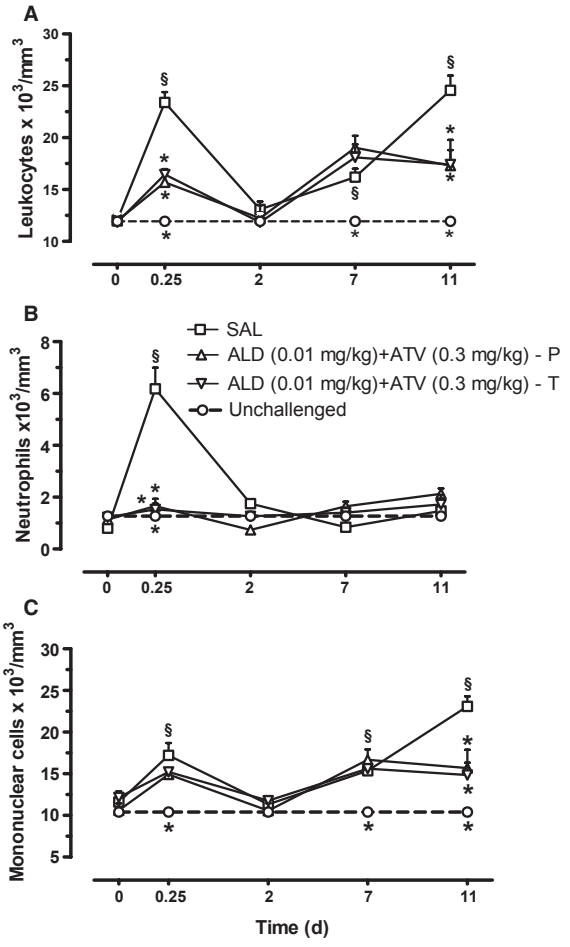

Fig. 4. Effect of prophylactic or therapeutic combinations of lower doses of alendronate (ALD) and atorvastatin (ATV) (0.01 mg/kg + 0.3 mg/kg, respectively) on leukocyte counts. The points represent the mean±standard error of the mean of total leukocytes (A), neutrophils (B) and mononuclear cells (C) $\times$ 10^3/mm^3 of a minimum of six rats per group. *Significant difference ($p < 0.05$) compared with the groups administered saline (SAL). §Significant difference ($p < 0.05$) compared with baseline. (Differences between mean values were calculated using ANOVA followed by the Bonferroni test.)

shown to be potent inhibitors of osteoclast-mediated bone resorption (3). Moreover, in addition to prevention of cardiovascular disease, statins have shown important pleiotropic effects, such as anti-inflammatory and antiresorptive effects (7).

We observed that when ALD or ATV was administered as monotherapy, only high doses were effective in preventing alveolar bone loss. The protective effects of these drugs are related to mechanisms that inhibit inflammation and bone resorption. ALD has shown anti-inflammatory activity through the reduction of IL-1β, IL-6 and tumor necrosis factor-alpha (21). ATV has been reported to inhibit IL-6 (22), inducible nitric oxide synthase immunostaining (10), cyclooxygenase-2 expression and MMP-9 activity (23).

Nitrogen-containing BPs exert a well-known bone-sparing effect on bone tissue by inhibition of farnesyl diphosphate synthase, a key enzyme in the mevalonate pathway, which results in a shortage of farnesyl diphosphate and geranylgeranyl pyrophosphate. This shortage prevents isoprenylation of small GTPases, which play a critical role in osteoclast-mediated bone resorption (3). Statins have been shown to have anabolic bone properties, preserving alveolar bone by stimulating vascular endothelial growth factor expression in osteoblasts (24) and bone morphogenetic protein-2 (25), which increases the RANKL/osteoprotegerin ratio (7). In addition, because ALD and ATV both act on the mevalonate pathway, they interfere simultaneously with cholesterol biosynthesis, resulting in the inhibition of cholesterol. This effect may also contribute to alveolar bone protection because cholesterol decreases osteoblast activity and consequently prevents bone mineralization (26).

Therefore, considering that BPs and statins each have antiresorptive and bone anabolic properties (26), we evaluated if the combination of these drugs affect bone metabolism (27). To our knowledge, this is the first time that the effect of the combination of ALD and ATV on alveolar bone loss has been reported.

None of the combinations of ALD+ATV completely prevented alveolar bone loss, as ATV and ALD act mainly on the mevalonate pathway and therefore other pathways not affected by these drugs could perpetuate inflammation. However, all combinations of ALD+ATV showed significant alveolar bone protection when compared with SAL or low-dose monotherapy. Among these combinations, the lower doses of drug showed bone preservation, which was not observed when these doses were administered as monotherapies. In addition, a lower-dose combination of ALD+ATV did not induce important systemic changes; therefore, we can infer that this lower-dose combination of ALD+ATV might be advantageous, not only by controlling the inflammation and bone loss underlying periodontitis, but also for systemic parameters. There is no evidence that these drugs are stable in solution because of a possible pharmaceutical interaction, and it is noteworthy that we used different routes of administration – ATV was administered by gavage and ALD was administered subcutaneously. In addition, the kinetics of metabolism of ATV occurs through cytochrome P450 (28), whereas ALD does not undergo such

metabolism, ranging from being deposited directly on the bone to being excreted in urine. In fact, it was observed that the interaction of the drugs was beneficial because of their different mechanisms of action. The finding of systemic safety was important because the literature has shown that monotherapy with high doses of ATV or ALD, for a prolonged period of time, may cause hepatic damage (10) and osteonecrosis of jaws (27), respectively, while rhabdomyolysis is a side effect related to the combination of ALD+ATV. It is worth pointing out, however, that such an effect occurs in a dose-dependent manner (29). It has been suggested that this effect could be caused by the potential of nitrogen-containing BPs to enhance the effect of statins to lower cholesterol, which could lead to abnormal membrane behavior, affect intracellular signaling and reduce mitochondrial respiratory function (30). Therefore, considering that this important side effect occurs in a dose–response manner (31), the use of low doses can minimize adverse events.

Prophylactic administration of a lower-dose combination of ALD and ATV showed significant bone protection when compared with SAL or low-dose monotherapy. When administered therapeutically, this combination also prevented alveolar bone loss. In the histometric study, the rats that were treated therapeutically showed an even higher degree of bone protection compared with those treated prophylactically, which is related to lower stress induced by manipulation or drug administration. Stress can increase bone loss by a local increase in proinflammatory (IL-1β, IL-6 and interferon-gamma) and proresorptive (RANKL) factors (32). Moreover, the number of rats used, as well as small variations of microscopy technique, may have contributed to this difference.

In this study, the histologic analysis showed that a lower-dose combination of ALD+ATV significantly preserved the alveolar bone and reduced inflammatory infiltrates. In inflammation, the initial step is migration of neutrophils to the challenged site. Then, neutrophils release enzymes such as MPO. Previously, an MPO assay has been used as an index of neutrophil infiltration and as a marker for acute inflammation in various injuries when polymorphonuclear cell infiltration occurs (33). According to our findings, the inflammatory infiltrates in periodontitis observed by histology are caused by neutrophil activity in the periodontal tissue. The reduction of MPO activity observed in the gingiva of rats treated with the lower-dose combination of ALD+ATV probably occurred because of a reduction of neutrophils in the area. In fact, the antioxidative effects of ALD have been previously reported. It has been shown that a nonchorinated BP, such as ALD, decreases MPO activity (34) and reduces neutrophil influx into rat gingiva subjected to periodontitis (35). ATV has been shown to improve abnormalities in the free-radical system and support the antioxidative defense mechanisms both *in vitro* and *in vivo* (36). Cadirci and colleagues (37) have shown that a reduction of MPO levels after ATV therapy is accompanied by a concomitant decrease in the activity of the antioxidant enzyme, superoxide dismutase. Statins also cause a dose-dependent inhibition in multiple steps of leukocyte recruitment and migration (38), which could reflect on MPO reduction (10).

We also observed that the lower-dose combination of ALD+ATV caused an important reduction of TRAP labeling. It has been described that TRAP is expressed by activated osteoclasts (39). Therefore, TRAP assays have often been used to assess bone resorption. We demonstrated that the lower-dose combination of ALD+ATV, administered prophylactically or therapeutically, markedly reduced TRAP expression. These results are in consistent with other studies that have shown a reduction of TRAP labeling after ALD therapy because ALD is internalized by osteoclasts and inhibits bone resorption by indirect prevention of protein isoprenylation, which causes osteoclast inactivation and apoptosis (40). Statins have also been shown to reduce the numbers of TRAP-positive multinucleated cells (41), indicating that the degree of bone formation is superior to that of bone resorption during low bone turnover in the groups treated with the lower-dose combination of ALD+ATV.

This study revealed that treatment with the lower-dose combination of ALD+ATV prevented the decrease of BALP after 11 d of ligature-induced alveolar bone loss. BALP is an enzyme that is highly expressed during osteoblastic differentiation and is concentrated on the membranes of matrix vesicles, which appear to be required for the initiation of mineralization (42). It is well documented that BPs, such as ALD, cause a reduction of BALP serum levels; however, this effect is dose-dependent because lower doses of BPs can stimulate BALP activity (43,44). ATV has been shown either to not alter or to slightly increase BALP serum levels (45), which could be explained by stimulation of bone morphogenetic protein-2 and induction of osteoblast differentiation (25).

Although ALD and ATV are drugs that are widely prescribed in clinical practice, we sought to evaluate the possible systemic implications of the use of the ALD + ATV combination. The lower-dose combination of ALD +ATV did not prevent the body mass loss observed in this study. Previous studies have shown that these drugs do not induce additional loss of weight (12), which indicates that this weight loss was probably caused by ligature trauma (11). Additionally, this combination of drugs administered prophylactically or therapeutically did not change the level of serum transaminases. In fact, it has been demonstrated that ALD is not metabolized in the liver (46). Studies in patients with liver transplants demonstrated that ALD was well tolerated without deleterious effects in liver function tests (AST and ALT) (47). The literature has reported that statins, including ATV, can induce an asymptomatic mild elevation of serum transaminases, but this elevation rarely requires withdrawal from the therapy (48). These studies support our idea of using the lower-dose combination of these drugs.

Regarding the leukogram changes, the lower-dose combination of ALD+ ATV also inhibited neutrophilia at 6 h after ligature placement and lymphomonocytosis on day 11 in treated rats. ALD has been shown to induce a significant decrease in total white blood cell, neutrophil and lymphocyte counts in patients with Paget's disease (49). ATV has also been shown to significantly reduce neutrophil migration (50). The recruitment and activation of polymorphonuclear neutrophils constitute the front line in the acute host inflammatory response, which represents the main source of prostaglandin E2 and promotes the initiation of bone metabolism breakdown by stimulating osteoclasts (51). Therefore, the ability to reduce neutrophilia appears to be important for reducing inflammatory bone loss. Reduction in the numbers of circulating mononuclear cells, which include monocytes, is also an important finding, considering that circulating monocytes might differentiate locally to osteoclasts and exert bone-resorbing activity (27). Thus, reduction in the numbers of mononuclear cells contributed to the bone-sparing effect of the lower-dose combination of ALD+ATV in this model. Additionally, oral treatment with ATV has been shown to reverse hematological changes induced by the inflammatory process (52).

In summary, rats subjected to periodontitis and treated with the lower-dose combination of ALD and ATV, administered prophylactically or therapeutically, showed a reduction of periodontal inflammation and alveolar bone loss without important systemic changes, which could be an interesting approach as an adjuvant treatment of periodontitis.

Acknowledgements

This work was supported by Brazilian grants from the National Council for Scientific and Techological Development - CNPq (grants 471407/2009-7), Coordination of Improvement of Higher Education Personnel - CAPES and Ceará foundation to support the development of scientific and technological - FUNCAP (Grants 247.01.00/09).

Conflict of interest

The authors declare no conflict of interest.

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