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Effect of alendronate on bone-specific alkaline phosphatase on periodontal bone loss in rats

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ABSTRACT

Objective: The study aims to evaluate the effect of alendronate (ALD) on bone-specific alkaline phosphatase (BALP) serum levels on periodontal bone loss in Wistar rats.
Design: Periodontitis was induced by ligature around the upper second molar in 36 male Wistar rats (±200 g). Groups of six animals received 0.9% saline (SAL) or ALD (0.01; 0.05; 0.25 mg kg^{-1}, s.c.), over 11 days; then they were sacrificed and their maxillae were removed to be defleshed and stained for macroscopic or histopathological analysis. Blood samples were collected for BALP, transaminases and total alkaline phosphatase (TALP) serum dosage, and haematologic study. Rats were weighed daily.
Results: Periodontitis induction caused reduction of BALP, intense alveolar bone loss (ABL), cementum and periodontal ligament destructions and intense leucocyte infiltration seen microscopically. Systemically, periodontitis induced leucocytosis, weight loss and TALP reduction. ALD (0.25 mg kg^{-1}) prevented BALP reduction (19.17 ± 1.36 U l^{-1}) when compared to SAL (13.6 ± 1.5), as well as prevented ABL, by 57.2%, when compared to SAL (4.80 + 0.18 mm^2), which was corroborated by histological findings (ALD 0.25 mg kg^{-1} = 1.5 (1–2) and SAL = 3 (2–3)) ($p < 0.05$). ALD did not alter transaminases but reduced TALP levels ($p < 0.05$). ALD 0.25 mg kg^{-1} reduced 6th-h neutrophilia (2.50 ± 0.22 cell × 10^3 mm^{-3}) and 7th- (12.29 ± 0.66) and 11th-day lymphomonocytosis (15.74 ± 0.52) when compared to SAL (5.20 ± 0.28; 18.24 ± 1.05; and 23.21 ± 1.48, respectively). ALD did not alter the weight loss.
Conclusion: ALD prevented BALP reduction and ABL and reduced inflammatory infiltrate, without causing systemic alterations.

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1. Introduction

Periodontitis is a chronic inflammatory disease of destructive and non-reversible action, that, if not treated, can cause tooth mobility leading to subsequent tooth loss.[1] Various mechanisms are related to aetiopathogenesis of periodontitis; however, factors associated to immunoinflammatory host response affect mainly development and use of drugs that might promote a modulation for this response.[1]

The mechanism of bone resorption in periodontitis is mediated by osteoclasts. These cells are originated by blood precursors from bone marrow, and are activated by various mediators, especially cytokines, such as tumour necrosis factor (TNF) and interleukin (IL)-1, which induce an increase of receptor activator of nuclear factor κ-B ligand (RANKL) on the osteoblast surface,[2] favouring RANK–RANKL linkage, which results in osteoclast activation and osteoclastogenesis.

On the resorption site, osteoclasts attach to the bone matrix through avβ1 integrin, forming a sealing zone.[3] Later,

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they organise their cytoskeleton, and then exhibit a ruffled border called the resorptive organ. By then, a great amount of acid vesicles are released on the resorption site, which are associated to a proton pump in order to start hydroxyapatite crystal dissolution.[3]

The nitrogen-containing bisphosphonates (nBPs) are pharmacological agents that possess a chemical structure similar to pyrophosphate, which provides a strong affinity to calcium. This structure promotes chelation to circulating calcium, binding it to the bone mineral surface.[4] Amongst bisphosphonates, sodium alendronate (ALD) stands out due to its high affinity to bone tissue.

The mechanism of action of nBP is based on the inhibition of the enzyme farnesyl diphosphate synthase (FPPS).[5] FPPS stimulates the isoprenylation of small guanosine-5′-triphosphatases (GTPases), which signalise to proteins that, when activated, regulate alterations on osteoclast morphology, cytoskeleton arrangement, vesicle traffic[5] and ruffled border. When the vesicular traffic and ruffled border are inhibited, the activities that elicit bone resorption are also reduced. Finally, when FPPS concentration reaches 100 μM, osteoclast apoptosis induction begins. Thus, nBPs are indicated as excellent bone resorption inhibitors.[5]

The enzyme alkaline phosphatase has been known for many years.[6] Alkaline phosphatase is a metalloenzyme anchored to the cell membrane, and it is distributed particularly in the liver, bowel, placenta and bone.[6] Bone-specific alkaline phosphatase (BALP), an isoenzyme of alkaline phosphatase, has been implicated in the processes of bone formation[6] and it is the major enzyme involved in removing inorganic pyrophosphate, an inhibitor of bone mineralisation.[6]

Because BALP is an exoenzyme that faces the extracellular compartment, it is conceivable that its activity and function can be modulated by environmental conditions.[6] Therefore, we aimed to evaluate the effect of ALD on BALP on periodontal bone loss in Wistar rats.

2. Materials and methods

2.1. *Animal selection*

Thirty-six male Wistar rats (*Rattus norvegicus*) weighing 180–220 g, from our own animal facilities, were used in this study. The animals were acclimatised for at least 1 week before the beginning of the experiment and were housed under normal laboratory conditions with laboratory chow and water available *ad libitum*. Experimental protocols were executed following ethical principles for laboratory animal use, and were approved by institutional Ethical Committee of Animal Research and European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Protocol no. 101/2009). All efforts were made to reduce animal number, their pain, suffering and stress.

2.2. *Model of experimental periodontitis*

The rats were divided into four groups, with six animals each. The model of ligature-induced periodontitis used consisted of insertion of nylon ligature around the cervix of second left upper molar of rats anaesthetised with chloral hydrate (Vetec®, Duque de Caxias, RJ, Brazil).[7,8] The ligature was placed through the proximal space of the respective tooth, and was knotted on the buccal side of the tooth, resulting in a subgingival position palatinally and in a supragingival position buccally of the ligature. The contralateral right side was used as the unligated control. Animals were observed until the 11th day, the period of the most intense alveolar bone loss, when they were then sacrificed. All ligature-induced periodontitis was made randomly.

2.3. *Experimental groups*

2.3.1. *Saline group*

This control group was constituted by six rats submitted to periodontitis. The animals received 0.5 ml of 0.9% sterile saline solution subcutaneously (s.c.), 30 min before ligature and, after that, daily, for an 11-day period, when they were then sacrificed.

2.3.2. *ALD groups*

The animals were subdivided in three groups of six animals each, which received ALD subcutaneously (Fosamax®, Merck, São Paulo-SP, Brazil) dissolved in 0.9% sterile saline solution in the doses of 0.01, 0.05 and 0.25 $mg\ kg^{-1}$, respectively, 30 min before ligature, and daily until the 11th day.

2.4. *Morphometric study of bone tissue*

On the 11th day, after periodontitis induction, the animals were sacrificed and their maxillae were removed and fixed in 10% neutral buffered formalin (Reagen®, Rio de Janeiro, RJ, Brazil), for 24 h. Following that, the maxillae were separated in half, dissected and stained with 1% aqueous methylene blue (Vetec®, Duque de Caxias, RJ, Brazil) and placed on microscope slides.[8,9] Then, they followed to photographic registration using a digital camera, Nikon® (D40, Melville, NY, USA). The measurement of the resorption area was made by a delimited region, involving the occlusal border of the vestibular side of the hemimaxilla until bone border. These areas were evaluated by ImageJ® software (Software ImageJ 1.32j, National Institutes of Health; EUA) in accordance to methodology described by Goes et al.[8]

2.5. *Histological analysis of alveolar bone*

Extra groups of six animals with periodontitis that had received saline or ALD (0.25 $mg\ kg^{-1}$) were sacrificed as described above and had their maxillae excised. The specimens were fixed in 10% neutral buffered formalin and were demineralised in 10% ethylene diamine tetraacetic acid (EDTA) (Dinâmica Química Contemporânea®, Diadema, SP, Brazil) for 40 days. Then, the specimens were dehydrated, embedded in paraffin and sectioned along the molars in a mesio-distal plane for Mallory trichrome staining. Sections of 4-μm thickness, corresponding to the area between the first and second molars, were evaluated by light microscopy. Parameters such as inflammatory cell infiltration, osteoclast number, alveolar bone and cementum integrity were determined in a single-blind manner and graded, by scores varying

from 0 to 3, based on the intensity of findings, as follows: Score 0: absence of or only discrete cellular infiltration, few osteoclasts, preserved alveolar process and cementum; Score 1: moderate cellular infiltration, presence of some osteoclasts, some but minor alveolar process resorption and intact cementum; Score 2: accentuated cellular infiltration, large number of osteoclasts, accentuated degradation of the alveolar process and partial destruction of cementum; and Score 3: accentuated cellular infiltrate and total destruction of alveolar process and cementum.[9]

2.6. *Serum dosage of BALP*

Blood samples were collected from the orbital plexus of anaesthetised animals (saline and ALD) before the experiment and on the 11th day. The BALP was evaluated using the thermoactivation method, by heating the sample at 56 °C for 10 min,[10] since BALP is a thermosensible isoform of total alkaline phosphatase (TALP). BALP serum levels were obtained by the subtraction of heated alkaline phosphatase from TALP serum levels. The methodology used to evaluate the enzymes' serum levels followed the manufacturers' directions (Labtest®, Lagoa Santa-MG, Brazil).

2.7. *Serum dosage of transaminases (AST and ALT) and TALP*

On the baseline and on the 11th day of the assay, blood samples were collected from the orbital plexuses of anaesthetised animals (saline and ALD). Liver function was evaluated through serum dosage of transaminases: aspartate aminotransferase (AST) and alanine aminotransferase (ALT). TALP serum levels were also evaluated. Specific kits were used, and methodology followed the manufacturer's instructions (Labtest®, Lagoa Santa-MG, Brazil).

2.8. *Haematologic study*

The method used to analyse white blood cell counts, as well as its subpopulation (neutrophil and mononuclear cells), was as follows: 20 μl of blood, taken from the rat tail, was added to 380 μl of Turk solution. Total white blood cell counts were performed using a Neubauer chamber and the differential counts were made using smears stained by rapid Instant Prov Stain Set (Newprov Produtos para Laboratório; Pinhais-PR, Brazil). A leucogram of the groups of animals (saline and ALD) was performed before periodontitis induction, at the 6th hour and 2nd, 7th and 11th days after the ligature.

2.9. *Corporal mass variation*

Animals from saline and ALD groups had their body mass measured before periodontitis induction and after that, daily until the 11th day. Values were expressed as body mass variation (g) compared to the initial body mass.

2.10. *Statistical analysis*

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

3. Results

3.1. *Morphometric study of bone tissue*

The macroscopic analysis of alveolar bone showed that 11 day ligature-induced periodontitis caused intense bone resorption (Table 1), associated with root exposition and furcation lesion (Fig. 1(d)). ALD, at the lowest dose (0.01 mg kg^{-1}), did not protect alveolar bone ($p > 0.05$) when compared to saline. ALD at higher doses (0.05 and 0.25 mg kg^{-1}) was able to significantly inhibit bone loss by 33.5% and 57.2%, respectively, when compared to saline ($p < 0.05$). Although the animals treated with ALD (0.25 mg kg^{-1}) had not presented alveolar bone preservation similar to normal hemimaxilla (Fig. 1(a)), the periodontal aspect was different from saline (Fig. 1(g)).

3.2. *Histological analysis of alveolar bone*

For the histological analysis, another assay was performed, and then the hemimaxillae were processed for histological analysis (Table 1). It was observed that alveolar bone and cementum resorptions were associated to an important inflammatory infiltrate ($p < 0.05$) on animals submitted to periodontitis (Table 1; Fig. 1(e) and (f)), when compared to normal periodontium (Table 1; Fig. 1(b) and (c)) ($p < 0.05$). ALD (0.25 mg kg^{-1}) treatment significantly attenuated the inflammatory infiltrate and preserved periodontal ligament, root cementum and alveolar bone (Table 1; Fig. 1(h) and (i)), when compared to saline ($p < 0.05$).

3.3. *Serum dosage of BALP*

Serum dosages of BALP were analysed (Fig. 2). Saline presented a significant decrease by 45.6% on BALP serum levels (13.62 ± 1.56 U l^{-1}) when compared to its baseline (25.04 ± 1.43 U l^{-1}). The treatment with ALD (0.01 and 0.05 mg kg^{-1}) caused a reduction of BALP serum levels, although not significant ($p > 0.05$), by 17.6% (19.92 ± 2.97 U l^{-1}) and 19.5% (21.62 ± 2.39 U l^{-1}), respectively, when compared to its respective baseline (ALD 0.01 = 24.19 ± 1.62; ALD 0.05 mg kg^{-1} = 26.67 ± 2.15 U l^{-1}). The treatment with ALD (0.25 mg kg^{-1}) induced a significant decrease by 28.1% (19.17 ± 1.36 U l^{-1}) for this enzyme after 11 days of ligature-induced periodontitis when compared to its baseline data (26.67 ± 2.15 U l^{-1}); however, the treatment with the highest dose of ALD prevented BALP reduction by 17.5%, when compared to saline after 11 days of periodontitis ($p < 0.05$).

3.4. *Serum dosage of transaminases and TALP*

Serum dosages of transaminases (AST and ALT) and TALP were analysed in animals of saline and ALD groups (Table 2).

Table 1 – Macroscopic and histological analysis of normal hemimaxilla or submitted to periodontitis receiving saline or ALD.

	Normal	Saline	ALD 0.01 mg kg^{-1}	ALD 0.05 mg kg^{-1}	ALD 0.25 mg kg^{-1}
Macroscopic analysis Mean ± SEM	–	4.8 ± 0.2	4.1 ± 0.4	3.2 ± 0.5*	2.1 ± 0.1*
Histological analysis Scores	0 (0–0)	3 (2–3)#	–	–	1.5 (1–2)*

(–) Indicates that there was no analysis. For macroscopic analysis, values represent the mean followed by S.E.M of a minimum of 6 animals per group by Anova and Bonferroni test. For histological analysis, values represent the medians followed by scores variation (lower–higher) of a minimum of 6 animals per group by Kruskal–Wallis and Dunn test.
* Statistically significant difference when compared to saline.
# Statistically significant difference when compared to normal hemimaxillae ($p < 0.05$).

On the 11th day, for AST and ALT, there was no statistical difference in the saline group when compared to its respective baseline. However, a significant decrease in TALP serum levels was observed in the animals from the saline group after 11 days, when compared to its baseline data. The treatment with ALD did not cause significant alteration ($p > 0.05$) in AST and ALT serum levels, but it reduced ($p < 0.05$) TALP serum levels when compared to its respective baseline data.

3.5. *Haematologic study*

Regarding total leucocyte counts, it was observed that periodontitis caused leucocytosis at the 6th hour after ligature

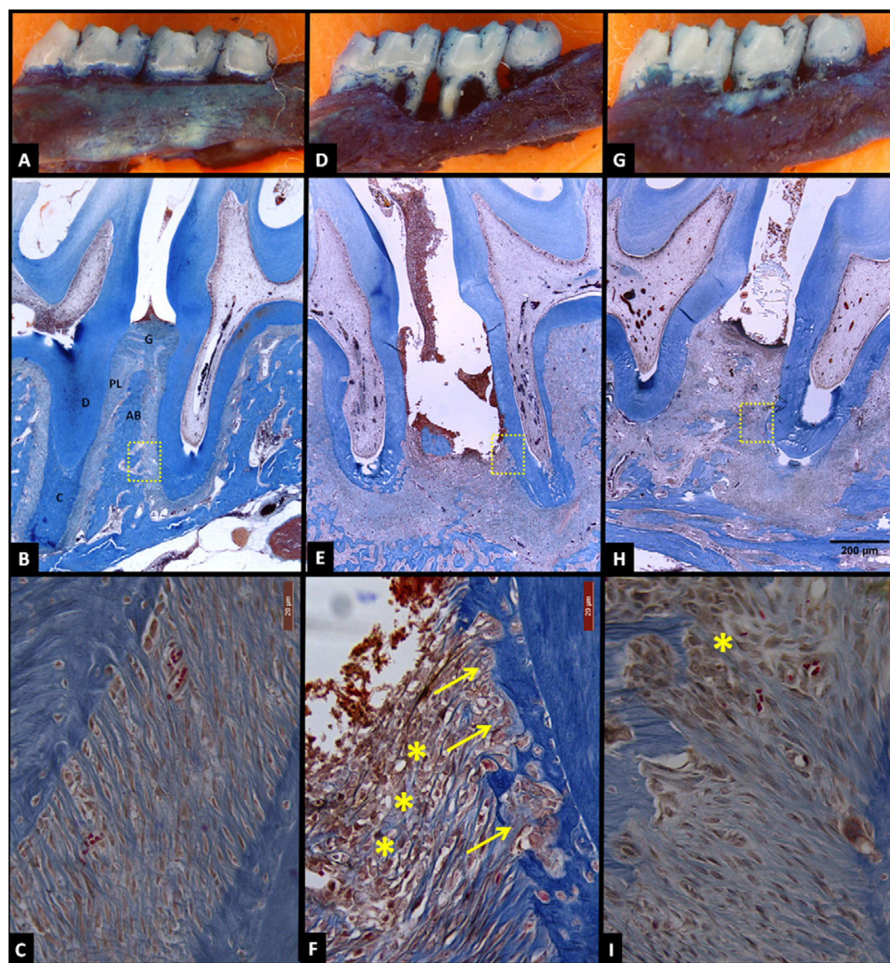

Fig. 1 – Macroscopic and microscopic aspect respectively of normal periodontium (A)–(C) and periodontium of rat submitted to periodontitis receiving saline, showing macroscopic bone resorption (D) and alveolar bone and cementum resorption, and inflammatory cell infiltration seen in histopathology (E) and (F). (G) (H) and (I) illustrate the reduction of inflammation and alveolar bone loss in periodontium of rats treated with ALD (0.25 mg kg^{-1}) for 11 days (macroscopic original magnification 7×; microscopic original magnification 40× and 400×). Letters of photomicrographs: G: gingiva; PL: periodontal ligament; D: dentin; AB: alveolar bone; C: cementum. Arrows indicate areas of resorption and (*) indicates inflammatory infiltrate. Dashed square areas (in B, E and H) indicate regions increased to 400× (in C, F and I).

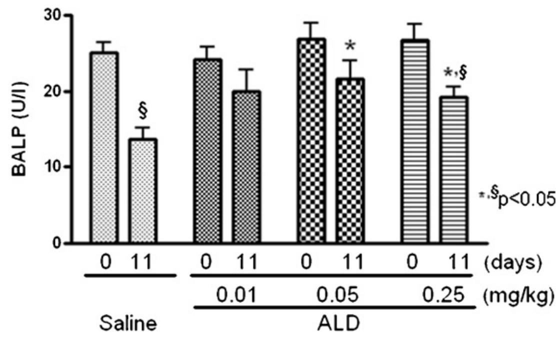

Fig. 2 – Effect of ALD on bone-specific alkaline phosphatase. Bars represent mean ± SEM of BALP (U/l) of a minimum of 6 animals per group. (*) indicates statistically significant difference when compared to saline 11 day data. (§) indicates statistically significant difference when compared to its respective baseline data [Two-way Anova; Bonferroni test and Student's *t*-test] ($p < 0.05$).

(18.77 ± 1.66 leucocytes $\times 10^3$ mm^{-3}) (Fig. 3(a)), when compared to its baseline data (11.56 ± 0.31). This leucocytosis was marked ($p < 0.05$) by neutrophilia (5.20 ± 0.28 neutrophil $\times 10^3$ mm^{-3}), when compared to its baseline (1.37 ± 0.08) (Fig. 3(b)). Following, on the 2nd day, there was a decrease in total leucocyte count; however, the basal cell counts were not achieved. New leucocytosis was observed on the 7th (21.73 ± 0.87 leucocytes $\times 10^3$ mm^{-3}) and 11th (25.84 ± 1.23) days, with predomination of mononuclear cells (7th day = 18.24 ± 1.05; 11th day = 23.21 ± 1.48 mononuclear cells $\times 10^3$ mm^{-3}) when compared to its baseline (10.19 ± 0.25) (Fig. 3(c)). All doses of ALD prevented neutrophilia at the 6th hour (ALD 0.01 = 4.00 ± 0.42; ALD 0.05 = 2.98 ± 0.21; ALD 0.25 = 2.50 ± 0.22), when compared to saline (5.20 ± 0.28) ($p < 0.05$) (Fig. 3(b)). However, only ALD (0.25 mg kg^{-1}) prevented mononuclear cell peaks on the 7th (12.29 ± 0.66) and 11th (15.74 ± 0.52) days (Fig. 3(c)).

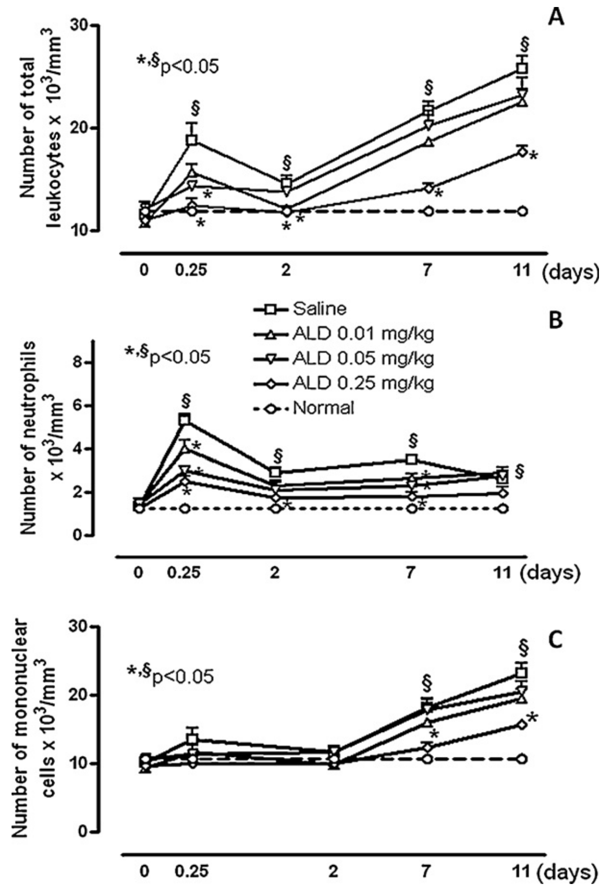

Fig. 3 – Effect of ALD on leukocyte counts. Points represent mean ± SEM of total leukocytes (A), neutrophils (B), mononuclear cells (C) × 10^3 mm^{-3} of a minimum of 6 animals per group. (*) indicates statistically significant difference when compared to saline. (§) indicates statistically significant difference when compared to its baseline data [Anova and Bonferroni test] ($p < 0.05$).

3.6. *Corporal mass variation*

Periodontitis caused body weight loss noted on the 3rd day after ligature placement when compared to normal animals. After that, animals showed gain of weight and a tendency to follow the normal animal corporal mass curve. Animals treated with ALD showed a similar corporal mass pattern to saline. ALD did not alter initial loss of weight, when compared to saline. After the 3rd day, gain of mass was observed accompanying animals from the saline group (Fig. 4).

Table 2 – Serum dosage of AST and ALT and TALP of animals submitted to periodontitis and receiving saline or ALD.

	Days	Groups Saline	ALD 0.01 mg kg^{-1}	ALD 0.05 mg kg^{-1}	ALD 0.25 mg kg^{-1}
AST (U/l)	0	44.51 ± 2.13	40.61 ± 2.97	45.44 ± 3.92	47.44 ± 3.33
	11	48.64 ± 4.74	38.72 ± 2.50	46.04 ± 3.86	42.51 ± 3.52
ALT (U/l)	0	18.44 ± 3.89	19.19 ± 3.81	17.36 ± 3.27	19.32 ± 4.18
	11	22.03 ± 3.44	19.91 ± 1.30	21.57 ± 2.72	16.02 ± 1.99
TALP (U/l)	0	95.61 ± 1.21	96.51 ± 1.52	97.07 ± 1.97	93.06 ± 1.09
	11	$70.14 \pm 1.74^{§}$	$77.29 \pm 1.99^{§}$	$75.75 \pm 2.11^{§}$	$69.64 \pm 1.71^{§}$

Values represent mean ± SEM of 6 animals per group.
§ Statistically significant difference when compared to its respective baseline data [Two-way Anova; Bonferroni test and Student's *t*-test] ($p < 0.05$).

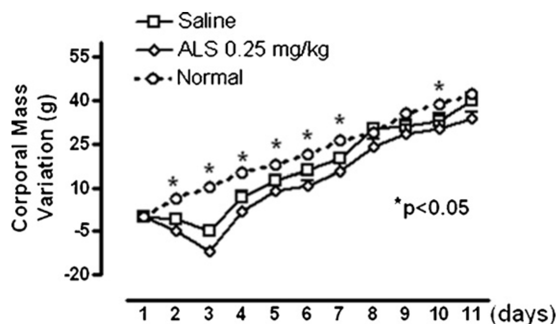

Fig. 4 – Effect of ALD on corporal mass variation. Points represent mean ± SEM of a minimum of 6 animals per group. (*) indicates statistically significant difference when compared to saline [Anova and Bonferroni's test] ($p < 0.05$).

4. Discussion

In the present study, it was seen that ligature-induced periodontitis caused intense alveolar bone resorption and periodontal inflammation, as demonstrated by macroscopic and histological analyses. In addition, a significant decrease in BALP and TALP serum levels was observed, and no change in AST and ALT serum levels. Periodontitis caused leucocytosis marked by neutrophilia at the 6th h and marked by lymphomonocytosis on the 7th and 11th days. In addition, an initial weight loss followed by tendency to accompany normal rat corporal mass curve was observed. Treatment with ALD prevented alveolar bone resorption of animals submitted to ligature-induced periodontitis, confirmed in macroscopic and histological analyses, when compared to saline. ALD, at the higher dose, prevented the reduction of BALP serum levels when compared to saline, and did not alter transaminases' serum levels. Besides, ALD prevented 6th-h neutrophilia, as well as lymphomonocytosis observed on the 7th and 11th days. ALD did not prevent the initial weight loss, although the animals had shown gain of corporal mass similar to saline corporal mass curve.

It has been described that ALD is rapidly eliminated from plasma, and mainly distributed to the bone, where about 60% of the dose is localised in bone tissue of rats.[11] Accordingly, Azuma et. al.[12] observed the concentration of [^{14}C]-alendronate in several bone tissues at various times after the 0.05 mg kg^{-1} IV dose. The maximal concentration in long bone and lumbar spine occurred about 8 and 24 h, respectively, after the IV dose, and the concentration of ALD in bone at 2 h varied in a dose-dependent manner, when 0.05–15 mg kg^{-1} of [^{14}C]-alendronate was injected IV. Furthermore, reports from the literature have shown that nBPs not only acted on osteoclast bone resorption, but also affected the behaviour and metabolism of other bone-related cells, such as osteoblasts, osteocytes and macrophages.[13,14] Therefore, we aimed to evaluate BALP serum levels after treatment with ALD. BALP, an isoform of TALP, acts specifically as a bone formation marker. Its mechanism of action is based on inorganic pyrophosphate hydrolysis, removing this osteogenic inhibitor, while it creates inorganic phosphate, required for the generation and deposition of hydroxyapatite.[15] BALP is secreted from osteoblast membrane toward matrix vesicles, allowing the mineralisation process to occur.[15] It is known that mammalian-tissue BALP is strongly activated by divalent cations such as Mg^{2+} and Zn^{2+}, and has an active site and contains two Zn^{2+} ions that stabilise its tertiary structure.[14] The intestinal and placental isoenzymes are less influenced by these cations.[16]

In this study, we have shown that the lowest doses of ALD (0.01 and 0.05 mg kg^{-1}) prevented the reduction of BALP serum levels, when compared to its baseline data. On the other hand, the highest dose of ALD (0.25 mg kg^{-1}) prevented BALP reduction when compared to saline after 11 days of periodontitis, but it was significantly different on BALP serum levels when compared to its baseline. Although slight, the lower level of BALP after treatment with ALD may be related to two aspects: the chemical structure, which is closely linked to the anti-resorptive effect of this drug, and its concentration.[17,18] nBPs, like ALD, have two radicals linked to the carbon atom, one, called R1 that has a hydroxyl group (–OH) and improves mineral affinity, and the other one, called R2, which increases nBP potency to inhibit bone resorption.[14] This chemical structure elicits the development of a structural motif called 'bone hook' that binds to the mineral by chelation of divalent cations.[18] Therefore, considering that BALP needs divalent cations to become activated and that the ALD bone hook reduces the offer of these cations, our present observations suggest that the highest dose of ALD inhibited BALP activity through divalent cation chelation within the bone hook structure. This suggestion is based on a previous report where BALP inhibition was reversed by an excess of Zn^{2+} or Mg^{2+}.[13]

However, it was seen that lower doses of ALD prevented BALP reduction while the highest dose did not, when compared to its respective baseline; therefore, we can infer that ALD may have a dose-dependent effect on BALP serum levels. In fact, reports from the literature had already confirmed our finding.[17,18] For Still et al.[17] at lower concentrations (10^{-9} to 10^{-7} M), ALD increased the formation of fibroblastic colonies, suggesting a mild anabolic effect. The treatment with high concentrations (10^{-4} M) of ALD caused a total inhibition of colony formation. It was also found that intermediate concentrations (10^{-6} M) of ALD decreased the formation of colonies displaying osteoblastic characteristics such as alkaline phosphatase expression, collagen accumulation and calcification. It was also observed by Vaisman et al.[18] that low doses of nBPs (10^{-10} to 10^{-5} M) stimulated BALP activity, whereas high concentrations (10^{-4} M) inhibited it. Levels of 10^{-4} M of ALD are estimated to be found *in vivo* at resorption lacunae in experimental animal models. Thus, our present observations are physiologically relevant in the context of a local action of nBPs used in the treatment of different bone diseases, such as periodontitis.

In order to corroborate BALP serum level results, we evaluated the bone-sparing action of ALD on morphometric and histological analyses. A significant bone protection was observed when the highest dose of ALD was used. The alveolar bone protection performed by ALD after ligature-induced periodontitis has been demonstrated in previous reports, in studies using the similar methodology.[19,20] This anti-resorptive effect may be explained by the attraction of ALD to the

bone and its interference on enzyme activity.[21,22] nBPs, like ALD inhibit FPPS, a mevalonate pathway enzyme responsible for isoprenylation of small GTPases, such as Rab, Rac, Ras and Rho.[23] These small GTPases are signalling proteins that, when activated, regulate several structural properties important for osteoclast function, including morphology, cytoskeletal arrangement, vesicular trafficking and membrane ruffling.[24,25] By the time that vesicular trafficking and membrane ruffling are inhibited bone resorption is also reduced, due to FPPS inhibition and consequent GTPases isoprenylation decrease. Therefore, FPPS inhibition seems to be responsible for the pharmacologic effects of the nBPs at tissue level.[26]

The macroscopic aspect was corroborated by histological analysis, demonstrating partial preservation of alveolar bone, cementum and periodontal ligament as well as reduction of inflammatory infiltrate in animals receiving ALD. Beyond the anti-resorptive action, ALD has shown anti-inflammatory activity, by inhibition of pro-inflammatory cytokines release, such as IL-1, IL-6 and TNF, and of nitric oxide (NO).[27–29] This anti-inflammatory activity may also rebound on ALD anti-resorptive action, since IL-1 and TNF, mainly stimulate expression of RANKL, a TNF family cytokine, which is essential for osteoclastogenesis induction.[30]

Treatment with ALD seemed to be safe. Animals treated with ALD showed initial weight loss, similar to saline, which may have been caused by ligature placement.[7,9] After that, it was seen that ALD therapy did not induce additional loss of weight, according to previous data.[20] ALD therapy did not cause significant changes in AST and ALT serum levels, suggesting that ALD does not interfere with liver function, which was expected, since this drug is not metabolised in the liver.[31] Studies in patients who received liver transplant demonstrated that ALD has been well tolerated without deleterious effects on liver function tests.[32] Patients taking ALD and diagnosed with primary biliary cirrhosis did not present significant hepatic effects regarding biochemical parameters of liver disease.[33] Our study also revealed significant inhibition of TALP serum levels after 11 days of periodontitis in animals receiving either saline or ALD. This inhibition may be due to the reduction of the bone isoform, since BALP represents about 90% of the TALP.[16]

We also observed that ALD prevented neutrophilia and lymphomonocytosis. These findings are in accordance with a previous report in which ALD treatment induced a significant decrease in total white blood cell, neutrophil and lymphocyte counts, in patients with Paget's disease.[34] The reduction in neutrophil count may effect neutrophil migration and activity, once it was seen that ALD decreased on neutrophil influx using a carrageenan-induced peritonitis model and reduced myeloperoxidase activity as well.[20] In addition, the reduction in peripheral mononuclear cells, which includes monocytes and lymphocytes, was also an important finding considering that circulating monocytes can migrate and differentiate locally on osteoclasts, thereby exerting bone resorption activity.[22] Thus, the reduction of mononuclear cells may contribute to the bone-sparing effect of ALD in this model.

In summary, our results demonstrated that ALD prevented BALP reduction and ABL, and reduced inflammatory infiltrate, without causing systemic alterations.

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Competing interests

None declared.

Ethical approval

The experimental protocols were executed following ethical principles for laboratory animal use in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, and they were approved by Institutional Ethical Committee of Animal Research (Process No. 101/2009).

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