J Periodont Res 2007; 42: 466–473

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2007.00970.x

Alendronate therapy in cyclosporine-induced alveolar bone loss in rats

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Spolidorio LC, Marcantonio E Jr, Spolidorio DMP, Nassar CA, Nassar PO, Marcantonio RA, Rossa C Jr. Alendronate therapy in cyclosporine-induced alveolar bone loss in rats. J Periodont Res 2007; 42: 466–473. © 2007 The Authors. Journal compilation © 2007 Blackwell Munksgaard

Background and Objective: Cyclosporine A is an immunosuppressive drug that is widely used in organ transplant patients as well as to treat a number of autoimmune conditions. Bone loss is reported as a significant side-effect of cyclosporine A use because this can result in serious morbidity of the patients. As we have shown that cyclosporine A-associated bone loss can also affect the alveolar bone, the purpose of this study was to evaluate the effect of the concomitant administration of alendronate on alveolar bone loss in a rat model.

Material and Methods: Forty Wistar rats (10 per group) were given cyclosporine A (10 mg/kg, daily), alendronate (0.3 mg/kg, weekly), or both cyclosporine A and alendronate, for 60 d. The control group received daily injections of sterile saline. The expression of proteins associated with bone turnover, including osteocalcin, alkaline phosphatase and tartrate-resistant acid phosphatase (TRAP), and also the calcium levels, were evaluated in the serum. Analysis of the bone volume, alveolar bone surface, the number of osteoblasts per bone surface and the number of osteoclasts per bone surface around the lower first molars was also performed.

Results: The results indicate that cyclosporine A treatment was associated with bone resorption, represented by a decrease in the bone volume, alveolar bone surface and the number of osteoblasts per bone surface and by an increase in the number of osteoclasts per bone surface and TRAP-5b. These effects were effectively counteracted by concomitant alendronate administration.

Conclusion: It is concluded that concomitant administration of alendronate can prevent cyclosporine A-associated alveolar bone loss.

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Key words: alendronate; alveolar bone loss; biochemical markers of bone turnover; cyclosporine

Accepted for publication October 22, 2006

Cyclosporine A is an immunosuppressive agent used in organ-transplant patients and in the treatment of various autoimmune disorders. Its immunosuppressive actions are mediated by a competitive inhibition of the cytosolic protein phosphatase, calcineurin (1,2). The side-effects of cyclosporine A therapy include gingival overgrowth, nephrotoxicity, hepatotoxicity and bone loss (3,4). Our research group has recently shown that rats treated with cyclosporine A present increased alveolar bone loss (5). Bone loss is a clinically important problem affecting from 6 to 15% of all patients in the first year after organ transplantation (6,7); however, there are no epidemiological data on alveolar bone loss in cyclosporine A-treated humans. On the other hand, studies in experimental animals have suggested that the use of cyclosporine A may influence bone healing around titanium implants (8), as well as the healing of both alveolar mucosa and bone in tooth-extraction sockets (9,10).

The latest generation of oral bisphosphonate drugs, including alendronate, has been approved for the prevention and treatment of osteoporosis. These medications are chemically absorbed into bone, decreasing osteoclast number and activity and thereby decreasing bone resorption. Currently, these are the most important antiresorptive agents indicated for the prevention and treatment of osteoporosis in postmenopausal women (11,12), osteogenesis imperfecta (13,14),

Paget's disease (15), glucocorticoid-induced osteoporosis, hypercalcemia derived from malignancy and various experimental models of osteoporosis (16–18). Bisphosphonates are generally well tolerated and associated with minimal adverse effects; however, some studies have suggested that intravenous bisphosphonate use is associated with osteonecrosis of the jaw (19–22). Considering the importance of cyclosporine A treatment for a number of medical conditions, and because bone loss is an important negative side-effect of cyclosporine A, we evaluated whether concomitant administration of alendronate would minimize cyclosporine A-associated alveolar bone loss. To this end we performed a stereometric analysis and also measured biochemical markers of bone resorption in the serum.

Material and methods

All experiments were approved by the Animal Experiment Ethics Committee of the School of Dentistry at Araraquara, Sao Paulo State University (UNESP). Forty male Wistar rats (*Rattus norvegicus albinus*), weighing between 90 and 100 g, were randomly distributed into four experimental groups comprising 10 animals each. The rats were kept in a room with controlled temperature (21 ± 1°C), humidity (65–70%) and a 12-h light–dark cycle. Animals were fed standard rat chow and water *ad libitum*. One group (the cyclosporine A group) was administered cyclosporine A (Sandimmun®; Novartis Pharma AG, Basileia, Switzerland), subcutaneously, daily (10 mg/kg of body weight) for 60 d. According to Wassef *et al.* (23), this dosage provides plasma peak and therapeutic concentrations of 1000 and 750 ng/mL, respectively. This dosage of cyclosporine A and period of treatment were previously shown by our research group to cause alveolar bone loss in rats (5,24). Another group (the alendronate group) of 10 animals received weekly subcutaneous injections of alendronate (Merh, Rahway, NJ, USA) at 0.3 mg/kg for 60 d. The subcutaneous route was chosen to avoid the variability and difficulty associated with oral gavage, and also because this method has been used successfully in previous studies (25,26). The dose was within the range found to be safe and effective for increasing bone density in rats (0.125–0.35 mg/kg/wk), whereas literature reports on the posology include both weekly or daily administrations (26–29). Another group of 10 animals was treated with both cyclosporine A and alendronate concomitantly (cyclosporine A + alendronate group) at the indicated doses, whereas the control group received daily subcutaneous injections of sterile saline (NaCl 0.9%). All animals were weighed weekly.

Blood collection

At the end of the experimental period on day 60, the animals were anesthetized with 0.1 mg/100 g body weight of Ketamine (Francotar®) and 0.04 mL/100 g of Xylazine (Virbaxil®) (Virbac do Brazil Ind. and Com. Ltda, Sao Paulo, SP, Brazil), and 5–6 mL of blood from the abdominal descending aorta of each rat was drawn into heparinized capillary tubes. The animals were killed humanely on day 60 by an overdose of anaesthetics.

Biochemical markers of bone formation and bone resorption in the serum

Calcium — The serum calcium was measured by the o-CPC colorimetric method using an ICA-1 ionized calcium analyzer (Radiometer Company, Copenhagen, Denmark).

Alkaline phosphatase — Total serum alkaline phosphatase activity was measured colorimetrically (ALP Kit-Sera Pak; Bayer AG, Elitech, France) using *para*-nitrophenyl phosphate as the substrate. Alkaline phosphatase activity was measured by the absorbance at 405 nm, using a Technicon SMA-24 spectrophotometer (Technicon, Domont, France). The units (U/dL) of enzyme activity in the experimental sample were calculated from this standard of Bayer units.

Osteocalcin — The osteocalcin concentration was determined in serum samples using the Rat-MID Osteocalcin enzyme-linked immunosorbent assay (ELISA) (Osteometer BioTech A/S, Herlev, Denmark). The Rat-MID Osteocalcin ELISA is based upon the competitive binding of a monoclonal antibody to osteocalcin. The monoclonal antibody is raised against human osteocalcin and recognizes the mid-part (amino acids 21–29) of the molecule. Synthetic human osteocalcin is used for standards. The test has been tested and proven to react with purified rat osteocalcin. The ELISA was performed using a Microplate Reader (ELx 800; Bio-Tek Instruments, Corp., Highland Park, Winooski, VT, USA). The coefficient of variation was 4.8%.

Osteoclast-derived tartrate-resistant acid phosphatase (TRAP-5b) — TRAP-5b was determined in the rat serum samples using a solid-phase immunofixed-enzyme activity assay (RatTrap Assay; SBA Sciences, Oulu, Finland). The RatTrap Assay uses a specific monoclonal antibody prepared using baculovirus-generated recombinant rat TRAP as the antigen. Serum samples are incubated with the antibody and bound TRAP-5b activity is determined at a pH where TRAP-5a is inactive and TRAP-5b is highly active, with a chromogenic substrate to develop color. Color intensity is directly proportional to the amount and activity of TRAP-5b in each sample. The assay results were analyzed using the Microplate Reader. The coefficient of variation was found to be 2.9%.

Tissue sections

After blood collection the rats were killed by an overdose of anaesthesia and the mandibles were carefully removed, dissected and fixed in 10% formalin for 48 h. Decalcification was carried out in 4.13% EDTA solution (pH 7.2) at 4°C for ≈3 mo. Serial paraffin sections of 5 μm were obtained in the buccal-lingual aspects of the whole first left and right molars and subsequently stained with hematoxylin and eosin. Morphological and stereological studies were made on the buccal, lingual and inter-radicular areas of alveolar bone (Fig. 1). Each lower first molar has a

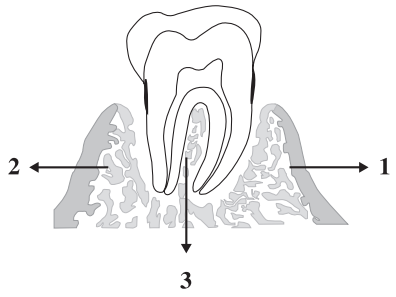

Fig. 1. Schematic illustration showing the regions where volumetric densities of the alveolar bone were made. 1, buccal bone region; 2, lingual bone region; 3, apical/inter-radicular bone region.

mesial-distal diameter of ≈1 mm, producing approximately 160 sections of 5 μm each.

Stereometric

The following stereometrical parameters were quantified, according to the methods used by Wada *et al*. (30) and Ogawa *et al*. (31). Nomenclature and abbreviations follow the recommendations of the American Society for Bone and Mineral Research (32). Bone volume (%) represents bone volume (mm^3) per total tissue volume (mm^3), the alveolar bone surface (mm^2/mm^3) represents alveolar bone surface (mm^2) per total tissue volume (mm^3), and osteoblasts surface and osteoclasts surface values indicate the number of osteoblasts and osteoclasts per bone surface, respectively. Measurements were performed with the aid of a Zeiss microscope (at a magnification of ×200) coupled to an Apple Macintosh SE computer and using a morphometry program named 'STEREOLOGY' (KSS Computer Engineer, Magma, UT). A total of 10–12 measurements were taken in each hemi-mandible to complete the stereometric analysis. The distance between the selected sections was 60 μm. Osteoclasts were defined as large, multinucleated cells attached to the bone surface. Osteoblasts were counted as ovoid cells lining the bone surface.

Statistical analysis

Measurements were expressed as mean and standard deviations of the data collected from all the animals in each group ($n = 10$). One-way analysis of variance was used for analysis of the effect of treatment groups on the variable. Whenever the factor group influenced the results, a posthoc Tukey test was used to determine pairwise differences between groups. Significance level was set to 5% ($p < 0.05$).

Results

All animals survived the whole experimental period. Cyclosporine A or alendronate administration, either isolated or combined, did not produce body weight alterations in comparison to untreated control animals.

Biochemical markers of bone formation and bone resorption in the serum

Calcium — In the control group, the mean serum calcium level was 10.4 ± 0.4 mg/dL. Cyclosporine A-treated groups showed a slight, nonsignificant ($p > 0.05$) decrease in the calcium levels. Treatment with alendronate alone, or with alendronate + cyclosporine A, also did not result in any change of the serum calcium levels ($p > 0.05$) (Fig. 2A).

Osteocalcin and TRAP-5b — In the control group, the mean osteocalcin level was 66.9 ± 0.3 ng/mL. Cyclosporine A treatment did not affect

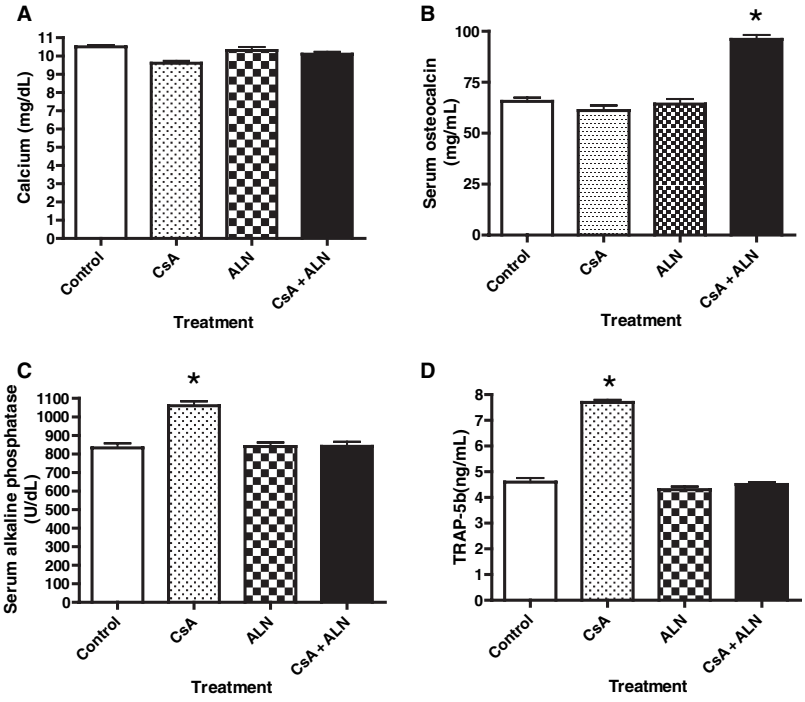

Fig. 2. Serum levels of bone turnover markers according to the treatment: control (untreated); cyclosporine A (10 mg/kg subcutaneously, daily, for 60 d); alendronate (0.3 mg/kg subcutaneously, weekly, for 60 d); and combined cyclosporine A and alendronate. (A) The calcium levels were slightly reduced by cyclosporine A treatment, whereas alendonate had no effect. (B) The osteocalcin level was also slightly reduced by treatment with cyclosporine A: even though osteocalcin was unchanged by alendonate alone, it was elevated by the treatement with cyclosporine A + alendonate. (C) Alkaline phosphatase levels were elevated only by cyclosporine A, as were the levels of osteoclast-derived tartrate-resistant acid phosphatase-5b (D). All values are means ± standard error of the mean, with $n = 10$ animals per experimental condition. *, $p < 0.05$ vs. the other groups. ALN, alendonate; CsA, cyclosporine A; CsA + ALN, cyclosporine A + alendonate; TRAP-5b, tartrate-resistant acid phosphatase-5b.

osteocalcin levels ($p > 0.05$). A slight and statistically nonsignificant decrease in osteocalcin levels was observed with alendronate treatment (53.8 ± 0.5 ng/mL; $p > 0.05$). On the other hand, combination treatment with cyclosporine A + alendronate induced a significant increase in serum osteocalcin (94.8 ± 0.7 ng/mL; $p < 0.05$) (Fig. 2B).

Alkaline phosphatase — The highest levels of serum alkaline phosphatase were observed in the cyclosporine A-treated group (Fig. 2C) ($p < 0.05$). In the control group the mean serum alkaline phosphatase level was 845 ± 62 U/dL, and in the cyclosporine A-treated group the mean serum alkaline phosphatase level was 957 ± 76 U/dL. The alendronate-treated and the cyclosporine A + alendronate combination treatment groups did not present significantly different levels of serum alkaline phosphatase when compared with the untreated control group ($p > 0.05$).

Osteoclast-derived TRAP-5b — In the control group, the mean TRAP-5b activity was 4.7 ± 0.3 U/L. The cyclosporine A-treated group demonstrated a significant increase in TRAP-5b activity (7.8 ± 0.4 U/L; $p < 0.05$). Alendronate treatment did not influence the activity of TRAP-5b (4.5 ± 0.6 U/L). Combined treatment with cyclosporine A + alendronate resulted in a TRAP-5b activity (4.7 ± 0.4 U/L) similar to that of the control and of the alendronate-treated groups (Fig. 2D).

Stereometric findings of alveolar bone

Tables 1 and 2 show the bone volume (%) and alveolar bone surface (mm^2/mm^3) from the buccal, apical and lingual regions of the first mandibular molars in each experimental group. Figure 3 shows typical micrographies of the decalcified transversal sections of the buccal, interradicular and lingual areas of the first mandibular from the control rats, rats treated with alendronate alone, rats treated with cyclosporine A only, and rats treated with cyclosporine A and alendronate at 60 d of treatment. Bone loss can be observed in rats treated with cyclosporine A (Figure 3A, B, C), and bone regeneration can be observed in rats treated with alendronate and cyclosporine A (Figure 4A, B, C). Figure 4 shows transversal sections of buccal area from rats treated with cyclosporine A. Osteoclasts can be seen which are large and have multiple nuclei evident on the bone surface. There was a significant ($p < 0.05$) decrease in bone volume and alveolar bone surface in cyclosporine A-treated animals in all analyzed regions in comparison to both control and alendronate-treated groups; however, the administration of alendronate and of alendronate + cyclosporine A increased the bone volume as well as the alveolar bone surface in comparison to the cyclosporine A-treated rats, in all analyzed areas ($p < 0.05$).

Table 1. Means and standard deviations for bone volume [which represents bone volume (mm^3) per total tissue volume (mm^3); %] from the buccal, apical and lingual regions of the first mandibular molar in control rats and in rats treated with cyclosporine A, alendonate, and cyclosporine A + alendonate

	Control	CsA	ALN	CsA + ALN
Buccal	1.93 ± 0.3	1.43 ± 0.2[a]	1.88 ± 0.3	1.81 ± 0.2
Apical	1.59 ± 0.3	1.48 ± 0.4[a]	1.85 ± 0.4	1.88 ± 0.3
Lingual	1.66 ± 0.2	1.45 ± 0.2[a]	1.81 ± 0.3	1.85 ± 0.3

[a]Significant difference from all other groups ($p < 0.05$) ($n = 10$ animals per group).
ALN, alendonate; CsA, cyclosporine A; CsA + ALN, cyclosporine A + alendonate.

Table 2. Means and standard deviations for the alveolar bone surface [which represents alveolar bone surface (mm^2) per total tissue volume (mm^3), mm^2/mm^3] from the buccal, apical and lingual regions of the first mandibular molar in control rats and in rats treated with cyclosporine A, alendonate, and cyclosporine A + alendonate

	Control	CsA	ALN	CsA + ALN
Buccal	20.4 ± 0.8	15.4 ± 1.2[a]	21.2 ± 0.9	20.1 ± 0.9
Apical	22.9 ± 0.7	14.1 ± 0.9[a]	21.9 ± 1.4	21.5 ± 0.8
Lingual	20.8 ± 1.2	15.3 ± 1.1[a]	20.1 ± 0.8	22.6 ± 1.3

[a]Significant difference from all other groups ($p < 0.05$) ($n = 10$ animals per group).
ALN, alendonate; CsA, cyclosporine A; CsA + ALN, cyclosporine A + alendonate.

Osteoblasts surface and osteoclasts surface

The osteoblasts surface and osteoclasts surface from the buccal, apical and lingual regions of the first lower molars in control and cyclosporine A-treated rats are shown in Tables 3 and 4. A significant decrease in the number of osteoblasts per bone surface and a significant increase in the number of osteoclasts per bone surface were observed in the cyclosporine A-treated groups, in comparison to the untreated controls ($p < 0.05$). Treatment with alendronate resulted in a small, nonsignificant increase in the number of osteoblasts per bone surface and did not alter the number of osteoclasts per bone surface in comparison with the untreated control group. However, combined treatment produced a

Table 3. Means and standard deviations for the number of osteoblasts per bone surface from the buccal, apical and lingual regions of the first mandibular molar in control rats and in rats treated with cyclosporine A, alendonate, and cyclosporine A + alendonate

	Control	CsA	ALN	CsA + ALN
Buccal	14.9 ± 3.6	10.9 ± 3.3[a]	15.3 ± 3.8	16.0 ± 3.4
Apical	15.6 ± 3.1	9.9 ± 2.5[a]	16.1 ± 3.5	15.9 ± 3.6
Lingual	14.8 ± 3.7	8.8 ± 2.3[a]	15.7 ± 3.9	15.8 ± 3.3

[a]Significant difference from all other groups ($p < 0.05$) ($n = 10$ animals per group).
ALN, alendonate; CsA, cyclosporine A; CsA + ALN, cyclosporine A + alendonate.

Table 4. Means and standard deviations for the number of osteoclasts per bone surface from the buccal, apical and lingual regions of the first mandibular molar in control rats and in rats treated with cyclosporine A, alendonate, and cyclosporine A + alendonate

	Control	CsA	ALN	CsA + ALN
Buccal	3.2 ± 2.1	14.3 ± 2.7[a]	3.3 ± 1.9	3.6 ± 2.6
Apical	3.1 ± 2.4	14.9 ± 4.1[a]	3.6 ± 1.4	3.4 ± 3.1
Lingual	2.9 ± 1.9	15.4 ± 4.2[a]	3.7 ± 2.7	4.5 ± 2.6

[a]Significant difference from all other groups ($p < 0.05$) ($n = 10$ animals per group).
ALN, alendonate; CsA, cyclosporine A; CsA + ALN, cyclosporine A + alendonate.

significant increase in the number of osteoblasts per bone surface and a significant decrease in the number of osteoclasts per bone surface ($p < 0.05$).

Discussion

In the present study, we evaluated the role of alendronate in the prevention of alveolar bone loss associated with cyclosporine A treatment in a well-characterized animal model. We could not find any studies regarding the effects of alendronate on cyclosporine A-induced alveolar bone in rats. The results of the present investigation indicate that concomitant administration of alendronate counteracted the bone resorption side-effects of cyclosporine A. In agreement with some reports (5,24,33), data confirmed that administration of cyclosporine A in immunosuppressive doses for 60 d resulted in evident gingival overgrowth (data not shown) and alveolar bone loss around the lower first molars. All the rats that were used in this experiment, despite being outbred, responded positively and uniformly to cyclosporine A, and this has also been reported by other authors. In humans, the incidence, as well as the progression, of cyclosporine A-induced bone loss seems to be variable. Although there is some controversy in the literature, there is accumulating evidence that in humans cyclosporine A has a negative impact on bone metabolism (3,5,30,34–39). Discrepancies in the data reported may be caused by differences in drug dosage, gender and age among the individuals included in the studies. Furthermore, the genetic capacity of the host to deal with administered drugs metabolically, and the individual responsiveness of bone to these drugs, may also hinder the evaluation of cyclosporine A-induced bone loss. In fact, cyclosporine A-induced gingival overgrowth and bone loss seem to be more uniform in rats than in humans. As commented by Kataoka *et al*. (17), many variables are better controlled in rats, such as genetic predisposition, gender, age, dose and duration of treatment with cyclosporine A.

Biochemical markers are important for the assessment of bone metabolism rate and have been used to evaluate the action of cyclosporine A on bone loss (30,40,41). Osteocalcin and alkaline phosphatase are markers of bone formation. Osteocalcin is expressed mainly in osteoblasts and alkaline phosphatase may be expressed in osteoblasts or hepatocytes (42,43). TRAP-5b is a resorption bone marker expressed mainly in osteoclasts (44). Expression of these bone markers is closely related to osteoblast and osteoclast differentiation and subsequent bone metabolism. Therefore, the increase of TRAP-5b observed in the present study may indicate that the cyclosporine A-induced alveolar bone loss is associated with metabolic changes of osteoclasts, inducing increased reabsorption of the alveolar bone. On the other hand, a small and statistically nonsignificant decrease in serum osteocalcin levels was observed. However, a decreased number of osteoblasts was detectable by histomorphometry. This finding is in line with the results of Wada *et al*. (30), who showed that the alterations in osteocalcin only occur before the bone loss when detected by hystomorphometry.

Only small and statistically nonsignificant changes in serum calcium levels were observed, which, according to Mason (45) and Ryffel (46), could be attributed to a nonspecific effect of cyclosporine A, resulting from modulation of renal excretion of calcium. In fact, other studies, using rat models treated with comparable immunosuppressive doses of cyclosporine A, have shown severe bone loss without any changes in ionized calcium (47,48). In accordance with a previous study from our group (5), a significant increase in serum alkaline phosphatase levels, a marker of osteoblast phenotype, was also observed with cyclosporine A treatment, suggesting a modest positive effect of cyclosporine A on bone formation. This effect could be the result of a negative feedback mechanism, because the stereometric analysis also confirmed previous results showing a decrease of bone volume, alveolar bone surface, a reduction of osteoblasts on the surface and an increase of osteoclasts on the surface. Buchinsky *et al*. (49) suggested that cyclosporine A may exert its osteopenic effect via the modulation of T cells rather than directly on bone cells. Thus, cyclosporine A may mediate its osteopenic effect by interfering in the cytokine expression by T cells, which will affect both osteoclasts and osteoblasts in the bone microenvironment (49,50), ultimately influencing bone remodelling (51). Paradoxically, cyclosporine A was recently shown to inhibit osteoclast formation by affecting calcineurin and subsequently the nuclear factor of activated T cells c1, which is a transcription factor associated with osteoclast differentiation and function (50).

The latest generation of bisphosphonate drugs, including alendronate, has been shown to inhibit bone loss both *in vitro* and *in vivo* (52–54). It has been suggested that these drugs induced minimal adverse effects; however, recent reports have suggested that intravenous bisphosphonate use is associated with osteonecrosis of the jaw (19–22). In contrast, Jeffcoat (55) showed that oral bisphosphonate use is not associated with the occurrence of osteonecrosis of the jaw. In the current study, alendronate was administrated subcutaneously and the dose of alendronate (3 μg) used corresponded to the human clinical dose (15 mg per person, orally) as the absorption rate is low in oral administration. After 60 d

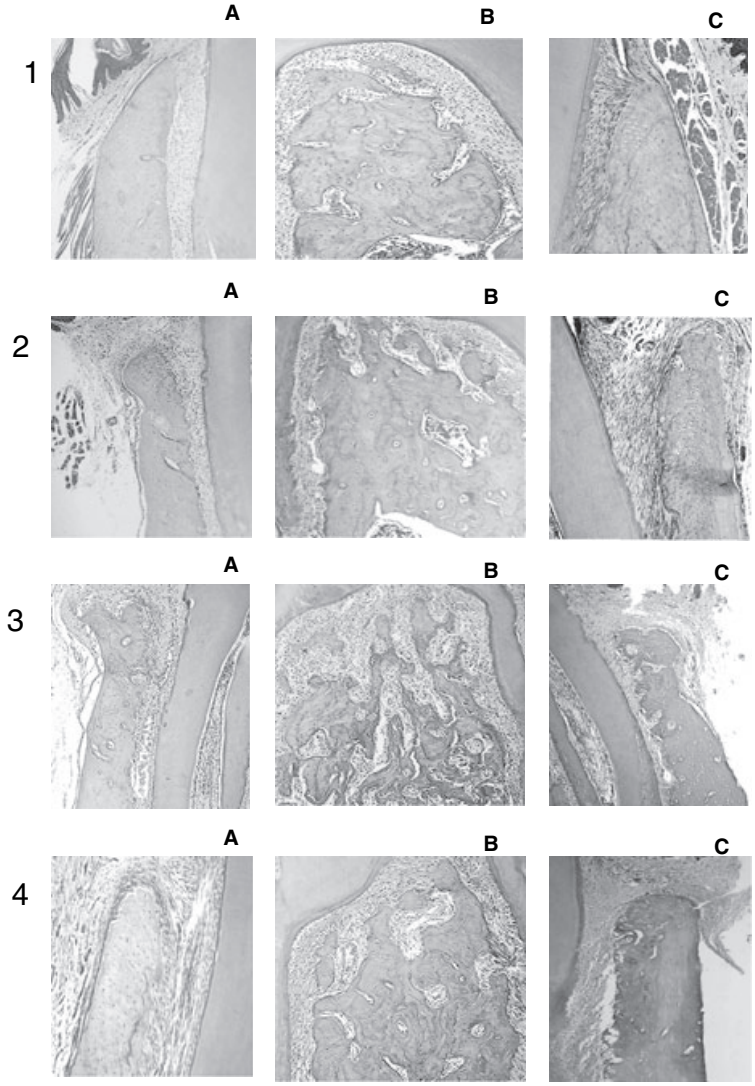

Fig. 3. Decalcified transversal sections of the buccal (A), interradicular (B) and lingual (C) region of the first mandibular molar, respectively, from controls (row 1), alendonate alone (row 2), cyclosporine A alone (row 3) and cyclosporine A + alendonate-treated rats (row 4) at 60 d of treatment. Bone loss was observed in cyclosporine A-treated rats (row 3, A,B,C), and bone regeneration was observed in alendonate + cyclosporine A-treated rats (row 4, A,B,C). (Hematoxylin and eosin stain, magnification ×40).

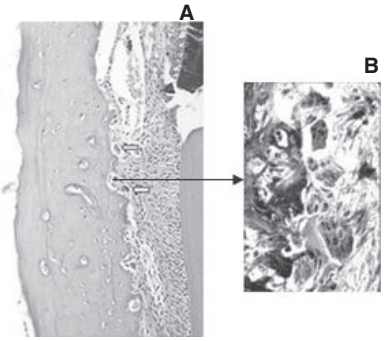

Fig. 4. Transversal sections of the buccal region from cyclosporine A-treated rats (A,B). Osteoclasts can be seen (arrows) (A) (hematoxylin and eosin stain, magnification ×40). Osteoclasts, which are large and have multiple nuclei, are evident on the bone surface (B). (Hematoxylin and eosin stain, magnification ×100).

of treatment, no ulcers were observed in the jaws of the rats, with only gingival overgrowth being detected. We found no studies to show the association between bisphosphonate and osteonecrosis of the jaw in rats. Although we did not observe any alteration in the jaws of the rats, we suggested that special attention should be paid to the patients treated with intravenous bisphosphonates. The understanding of the effects of these drugs on bone metabolim affected by cyclosporine A, if not of immediate clinical applicability, may provide cues for developing new compounds or therapeutic strategies to prevent this morbidity associated with cyclosporine A-induced bone loss.

Biochemical and histomorphometrical analysis data presented herein suggest that alendronate compensated the cyclosporine A-induced alveolar bone loss. With regard to the serum bone markers, a significant decrease in TRAP-5b and an increase in both osteocalcin and alkaline phosphatase were observed, suggesting an enhanced bone formation associated with an increased number of osteoblasts and a decreased number of osteoclasts when the treatments were associated. Interestingly, alendronate alone did not modulate bone turnover, as evaluated in this study. In fact, many reports suggest that bisphosphonates are chemically absorbed into bone, decreasing osteoclast number and activity and thereby decreasing bone resorption (27,56–68). Several reports have demonstrated that bisphosphonates induce the osteoblasts to secrete inhibitors of osteoclast-mediated resorption and also stimulate the formation of osteoblast precursors (57,58). Thompson *et al.* (59) recently reported that, by virtue of their ability to bind to Ca^{+} ions, bisphosphonates rapidly localize to bone mineral *in vivo* and accumulate beneath bone-resorbing osteoclasts (27,60) that subsequently release and internalize the bisphosphonates in the acidic environment of the resorption lacuna (69). In addition to these positive effects of alendronate on bone formation, conflicting data exist. Moreno *et al.* (37) related that alendronate *in vitro* does not affect the viability, proliferation and mineral deposit capacity of human osteoblasts.

We may speculate that alendronate has an effect on bone turnover only in situations of increased bone loss. In such situations, alendronate would increase bone mass by allowing formation to exceed resorption, thus

generating a net positive balance in bone mineral content (58,70,71). This possibility is supported by the results of studies on the therapeutic use of alendronate in osteoporotic patients, in which the progressive increase in bone mass observed was related to the modulation of bone turnover (70,72,73).

Within the limitations of our experimental model, we suggest that the alveolar bone loss associated with cyclosporine A administration may be minimized by concomitant treatment with alendronate. However, one has to consider the possible consequences of this combined treatment, especially the cost–benefit in terms of potential prejudicial side-effects of alendronate. To reduce the risk of osteonecrosis, patients should be evaluated by a dentist before starting treatment with intravenous bisphosphonates. Further studies are being designed and conducted on cells that are relevant for bone turnover, to address the mechanisms of action of both cyclosporine A and alendronate.

References

1. Faulds D, Goa KL, Benfield P. Cyclosporin. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in immunoregulatory disorders. *Drugs* 1993;**45:**953–1040.
2. Dunn CJ, Wagstaff AJ, Perry CM, Plosker GL, Goa KL. Cyclosporin: an updated review of the pharmacokinetic properties, clinical efficacy and tolerability of a microemulsion-based formulation (neoral) 1 in organ transplantation. *Drugs* 2001;**61:**1957–2016.
3. Marcen R, Cabarello C, Pascual J *et al.* Lumbar bone mineral density in renal transplant patients on neoral and tacrolimus: a four-year prospective study. *Transplantation* 2006;**81:**826–831.
4. Spolidorio LC, Spolidorio DM, Massucato EM, Neppelenbroek KH, Campanha NH, Sanches MH. Oral health in renal transplant recipients administered cyclosporin A or tacrolimus. *Oral Dis* 2006;**12:**309–314.
5. Spolidorio LC, Spolidorio DM, Holzhausen M. Effects of long-term cyclosporin therapy on the periodontium of rats. *J Periodont Res* 2004;**39:**257–262.
6. Epstein S, Shane E, Bilezikian JP. Organ transplantation and osteoporosis. *Curr Opin Rheumatol* 1995;**7:**255–261.
7. Cohen A, Shane E. Osteoporosis after solid organ and bone marrow transplantation. *Osteoporos Int* 2003;**14:**617–630.
8. Duarte PM, Nogueira Filho GR, Sallum EA, de Toledo S, Sallum AW, Nociti FH Jr. The effect of an immunosuppressive therapy and its withdrawal on bone healing around titanium implants. A histometric study in rabbits. *J Periodontol* 2001;**72:**1391–1397.
9. Silva HC, Coletta RD, Jorge J, Bolzani G, de Almeida OP, Graner E. The effect of cyclosporin A on the activity of matrix metalloproteinases during the healing of rat molar extraction wounds. *Arch Oral Biol* 2001;**46:**875–879.
10. Gau CH, Hsieh YD, Shen EC, Lee S, Chiang CY, Fu E. Healing following tooth extraction in cyclosporine-fed rats. *Int J Oral Maxillofac Surg* 2005;**34:**782–788.
11. Reginster JY, Sarlet N. The treatment of severe postmenopausal osteoporosis: a review of current and emerging therapeutic options. *Treat Endocrinol* 2006;**5:**15–23.
12. Hochberg MC, Rizzoli R. Long-term experience with alendronate in the treatment of osteoporosis. *Expert Opin Pharmacother* 2006;**7:**1201–1210.
13. Madenci E, Yilmaz K, Yihmaz M, Coskun Y. Alendronate treatment in osteogenesis imperfecta. *J Clin Rheumatol* 2006;**12:**53–56.
14. Sen C *et al.* Effects of calcitonin and alendronate on distraction osteogenesis. *Int Orthop* 2006;**30:**272–277.
15. Liel Y. Paget's disease and bisphosphonates. *N Engl J Med* 2005;**353:**2616–2618; author reply 2616–8.
16. Newman ED, Matzko CK, Olenginski TP *et al.* Glucocorticoid-Induced Osteoporosis Program (GIOP): a novel, comprehensive, and highly successful care program with improved outcomes at 1 year. *Osteoporos Int* 2006;**17:**1706.
17. Curtis JR, Westfall AO, Allison JJ, Freeman A, Saag KG. Channeling and adherence with alendronate and risedronate among chronic glucocorticoid users. *Osteoporos Int* 2006;**17:**1268–1274.
18. Gass M, Dawson-Hughes B. Preventing osteoporosis-related fractures: an overview. *Am J Med* 2006;**119:**S3–S11.
19. Delibasi T, Altundag K, Kanlioglu Y. Why osteonecrosis of the jaw after bisphosphonates treatment is more frequent in multiple myeloma than in solid tumors. *J Oral Maxillofac Surg* 2006;**64:**995–996.
20. Ruggiero SL, Fantasia J, Carlson E. Bisphosphonate-related osteonecrosis of the jaw: background and guidelines for diagnosis, staging and management. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2006;**102:**433–441.
21. Van Poznak C, Estilo C. Osteonecrosis of the jaw in cancer patients receiving IV bisphosphonates. *Oncology (Williston Park)* 2006;**20:**1053–1062; discussion 1065–1066.
22. Scully C, Madrid C, Bagan J. Dental endosseous implants in patients on bisphosphonate therapy. *Implant Dent* 2006;**15:**212–218.
23. Wassef R, Cohen Z, Langer B. Pharmacokinetic profiles of cyclosporine in rats. Influence of route of administration and dosage. *Transplantation* 1985;**40:**489–493.
24. Nassar CA, Nassar PO, Abi Rached RS, Holzhausen M, Marcantonio E Jr, Spolidorio LC. Effect of cyclosporin A on alveolar bone homeostasis in a rat periodontitis model. *J Periodont Res* 2004;**39:**143–148.
25. Bikle DD, Morey-Holton ER, Doty SB, Currier PA, Tanner SJ, Halloran BP. Alendronate increases skeletal mass of growing rats during unloading by inhibiting resorption of calcified cartilage. *J Bone Miner Res* 1994;**9:**1777–1787.
26. Camacho NP, Raggio CL, Doty SB *et al.* A controlled study of the effects of alendronate in a growing mouse model of osteogenesis imperfecta. *Calcif Tissue Int* 2001;**69:**94–101.
27. Azuma Y, Oue Y, Kanatani H, Ohta T, Kiyoki M, Komoriya K. Effects of continuous alendronate treatment on bone mass and mechanical properties in ovariectomized rats: comparison with pamidronate and etidronate in growing rats. *J Pharmacol Exp Ther* 1998;**286:**128–135.
28. Evans KD, Lau ST, Oberbauer AM, Martin RB. Alendronate affects long bone length and growth plate morphology in the oim mouse model for Osteogenesis Imperfecta. *Bone* 2003;**32:**268–274.
29. Spadaro JA, Damron TA, Horton JA, Margulies BS, Murray GM, Clemente DA, Strauss JA. Density and structural changes in the bone of growing rats after weekly alendronate administration with and without a methotrexate challenge. *J Orthop Res* 2006;**24:**936–944.
30. Wada C *et al.* High-turnover osteoporosis is induced by cyclosporin A in rats. *J Bone Miner Metab* 2006;**24:**199–205.
31. Ogawa K *et al.* Effects of combined elcatonin and alendronate treatment on the architecture and strength of bone in ovariectomized rats. *J Bone Miner Metab* 2005;**23:**351–358.
32. Parfitt AM. Bone histomorphometry: standardization of nomenclature, symbols and units (summary of proposed system). *Bone* 1988;**9:**67–69.
33. Cunningham J. Posttransplantation bone disease. *Transplantation* 2005;**79:**629–634.
34. Epstein S *et al.* Transforming growth factor-beta administration modifies cyclosporine A-induced bone loss. *Bone* 2001;**28:**583–588.

35. Ugur A, Guvener N, Isiklar I, Turan M, Erdal R, Haberal M. Osteoporosis after renal transplantation: single center experience. *Transplantation* 2001;**71:** 645–649.
36. Grotz W, Wanner C, Rother E, Scholmeyer P. Clinical course of patients with antineutrophil cytoplasm antibody positive vasculitis after kidney transplantation. *Nephron* 1995;**69:**234–236.
37. Moreno M, Manzanares C, Castellano F *et al*. Monitoring of tacrolimus as rescue therapy in pediatric liver transplantation. *Ther Drug Monit* 1998;**20:**376–379.
38. Loinaz C, Marquez E, Gomez R *et al*. Clinical features of 32 patients after 8 years of a liver transplant. *Transplant Proc* 1999;**31:**2475–2476.
39. Pichette V, Bonnardeaux A, Prudhomme L, Gagne M, Cardinal J, Ouimet D. Long-term bone loss in kidney transplant recipients: a cross-sectional and longitudinal study. *Am J Kidney Dis* 1996;**28:**105–114.
40. Kataoka M *et al*. Cyclosporin A decreases the degradation of type I collagen in rat gingival overgrowth. *J Cell Physiol* 2000;**182:**351–358.
41. Cunningham J. Posttransplantation bone disease. *Transplantation* 2005;**79:** 629–634.
42. Yao KL, Todescan R Jr, Sodek J. Temporal changes in matrix protein synthesis and mRNA expression during mineralized tissue formation by adult rat bone marrow cells in culture. *J Bone Miner Res* 1994;**9:**231–240.
43. Ikedo D *et al*. Stimulatory effects of phenytoin on osteoblastic differentiation of fetal rat calvaria cells in culture. *Bone* 1999;**25:**653–660.
44. Igarashi Y, Lee MY, Matsuzaki S. Acid phosphatases as markers of bone metabolism. *J Chromatogr B Analyt Technol Biomed Life Sci* 2002;**781:**345–358.
45. Mason J. Renal side-effects of cyclosporin A. *Br J Dermatol* 1990;**122:**71–77.
46. Ryffel B. Cyclosporin. Toxicology – experimental studies. *Prog Allergy* 1986;**38:**181–197.
47. Movsowitz C, Epstein S, Fallon M, Ismail F, Thomas S. Cyclosporin-A in vivo produces severe osteopenia in the rat: effect of dose and duration of administration. *Endocrinology* 1988;**123:**2571–2577.
48. Rucinski B, Liu CC, Epstein S. Utilization of cyclosporine H to elucidate the possible mechanisms of cyclosporine A-induced osteopenia in the rat. *Metabolism* 1994;**43:**1114–1118.
49. Buchinsky FJ, Ma Y, Mann GN *et al*. Bone mineral metabolism in T lymphocyte-deficient and -replete strains of rat. *J Bone Miner Res* 1995;**10:**1556–1565.
50. Chowdhury MH, Shen V, Dempster DW. Effects of cyclosporine A on chick osteoclasts *in vitro*. *Calcif Tissue Int* 1991;**49:**275–279.
51. McCauley LK, Rosol TJ, Capen CC. Effects of cyclosporin A on rat osteoblasts (ROS 17/2.8 cells) in vitro. *Calcif Tissue Int* 1992;**51:**291–297.
52. Sass DA, Bownam AR, Yuan Z, Ma Y, Jee WS, Epstein S. Alendronate prevents cyclosporin A-induced osteopenia in the rat. *Bone* 1997;**21:**65–70.
53. Altundal H, Gursoy B. The influence of alendronate on bone formation after autogenous free bone grafting in rats. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2005;**99:**285–291.
54. Ogawa K *et al*. Effects of combined elcatonin and alendronate treatment on the architecture and strength of bone in ovariectomized rats. *J Bone Miner Metab* 2005;**23:**351–358.
55. Jeffcoat MK. Safety of oral bisphosphonates: controlled studies on alveolar bone. *Int J Oral Maxillofac Implants* 2006;**21:**349–353.
56. D'Aoust P, McCulloch CA, Tenenbaum HC, Lekic PC. Etidronate (HEBP) promotes osteoblast differentiation and wound closure in rat calvaria. *Cell Tissue Res* 2000;**302:**353–363.
57. Vitte C, Fleisch H, Guenther HL. Bisphosphonates induce osteoblasts to secrete an inhibitor of osteoclast-mediated resorption. *Endocrinology* 1996;**137:**2324–2333.
58. Giuliani N, Pedrazzpni M, Negri G, Passeri G, Impicciatore M, Girasole G. Bisphosphonates stimulate formation of osteoblast precursors and mineralized nodules in murine and human bone marrow cultures *in vitro* and promote early osteoblastogenesis in young and aged mice *in vivo*. *Bone* 1998;**22:**455–461.
59. Thompson K *et al*. Cytosolic entry of bisphosphonate drugs requires acidification of vesicles after fluid-phase endocytosis. *Mol Pharmacol* 2006;**69:**1624–1632.
60. Masarachia P, Weinreb M, Balena R, Rodan GA. Comparison of the distribution of 3H-alendronate and 3H-etidronate in rat and mouse bones. *Bone* 1996;**19:**281–290.
61. Benford HL, Frith JC, Auriola S, Monkkonen J, Rogers MJ. Farnesol and geranylgeraniol prevent activation of caspases by aminobisphosphonates: biochemical evidence for two distinct pharmacological classes of bisphosphonate drugs. *Mol Pharmacol* 1999;**56:**131–140.
62. van Beek E, Pieterman F, Cohen L, Lowik C, Papapoulos S. Farnesyl pyrophosphate synthase is the molecular target of nitrogen-containing bisphosphonates. *Biochem Biophys Res Commun* 1999;**264:**108–111.
63. Bergstrom JD, Bostedor RG, Masarachia PJ, Reszka AA, Rodan G. Alendronate is a specific, nanomolar inhibitor of farnesyl diphosphate synthase. *Arch Biochem Biophys* 2000;**373:**231–241.
64. Dunford JE, Thompson K, Coxon FP *et al*. Structure–activity relationships for inhibition of farnesyl diphosphate synthase *in vitro* and inhibition of bone resorption *in vivo* by nitrogen-containing bisphosphonates. *J Pharmacol Exp Ther* 2001;**296:**235–242.
65. Luckman SP, Hughes DE, Coxon FP, Graham R, Russell G, Rogers MJ. Nitrogen-containing bisphosphonates inhibit the mevalonate pathway and prevent post-translational prenylation of GTP-binding proteins, including Ras. *J Bone Miner Res* 1998;**13:**581–589.
66. Rogers MJ. New insights into the molecular mechanisms of action of bisphosphonates. *Curr Pharm Des* 2003;**9:**2643–2658.
67. Auriola S, Frith J, Rogers MJ, Koivuniemi A, Monkkonen J. Identification of adenine nucleotide-containing metabolites of bisphosphonate drugs using ion-pair liquid chromatography-electrospray mass spectrometry. *J Chromatogr B Biomed Sci Appl* 1997;**704:**187–195.
68. Frith JC, Monkkonen J, Auriola S, Monkkonen H, Rogers MJ. The molecular mechanism of action of the antiresorptive and antiinflammatory drug clodronate: evidence for the formation *in vivo* of a metabolite that inhibits bone resorption and causes osteoclast and macrophage apoptosis. *Arthritis Rheum* 2001;**44:**2201–2210.
69. Sato M, Grasser W. Effects of bisphosphonates on isolated rat osteoclasts as examined by reflected light microscopy. *J Bone Miner Res* 1990;**5:**31–40.
70. Tucci JR, Tonino RP, Emkey RD, Peverly CA, Kher U, Santora AC 2nd. Effect of three years of oral alendronate treatment in postmenopausal women with osteoporosis. *Am J Med* 1996;**101:**488–501.
71. Chavassieux PM, Arlot ME, Roux JP *et al*. Effects of alendronate on bone quality and remodeling in glucocorticoid-induced osteoporosis: a histomorphometric analysis of transiliac biopsies. *J Bone Miner Res* 2000;**15:**754–762.
72. Devogelaer JP, Broll H, Correa-Rotter R *et al*. Oral alendronate induces progressive increases in bone mass of the spine, hip, and total body over 3 years in postmenopausal women with osteoporosis. *Bone* 1996;**18:**141–150.
73. Frolik CA, Bryant HU, Black EC, Magee DE, Chandrasekhar S. Time-dependent changes in biochemical bone markers and serum cholesterol in ovariectomized rats: effects of raloxifene HCl, tamoxifen, estrogen, and alendronate. *Bone* 1996;**18:**621–627.