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Simvastatin therapy in cyclosporine A-induced alveolar bone loss in rats

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Background and Objective: Cyclosporine A treatment is important in the therapy of a number of medical conditions; however, alveolar bone loss is an important negative side-effect of this drug. As such, we evaluated whether concomitant administration of simvastatin would minimize cyclosporine A-associated alveolar bone loss in rats subjected, or not, to experimental periodontal disease.

Material and Methods: Groups of 10 rats each were treated with cyclosporine A (10 mg/kg/day), simvastatin (20 mg/kg/day), cyclosporine A and simvastatin concurrently (cyclosporine A/simvastatin) or vehicle for 30 days. Four other groups of 10 rats each received a cotton ligature around the lower first molar and were treated similarly with cyclosporine A, simvastatin, cyclosporine A/simvastatin or vehicle. Calcium (Ca^{2+}), phosphorus and alkaline phosphatase levels were evaluated in serum. Expression levels of interleukin-1β, prostaglandin E_2 and inducible nitric oxide synthase were evaluated in the gingivomucosal tissues. Bone volume and numbers of osteoblasts and osteoclasts were also analyzed.

Results: Treatment with cyclosporine A in rats, with or without ligature, was associated with bone loss, represented by a lower bone volume and an increase in the number of osteoclasts. Treatment with cyclosporine A was associated with bone resorption, whereas simvastatin treatment improved cyclosporine A-associated alveolar bone loss in all parameters studied. In addition, simvastatin, in the presence of inflammation, can act as an anti-inflammatory agent.

Conclusion: This study shows that simvastatin therapy leads to a reversal of the cyclosporine A-induced bone loss, which may be mediated by downregulation of interleukin-1β and prostaglandin E_2 production.

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Osteoporosis is a significant adverse reaction in transplant recipients. Recently, an increasing number of immunosuppressive programmes use glucocorticoid-free regimens, but other immunosuppressants, such as calcineurin inhibitors (e.g. cyclosporine A), are also associated with the pathogenesis of transplantation-related osteoporosis (1–3). We have shown that cyclosporine A therapy in rats affects alveolar bone and that the deleterious periodontal effects of cyclosporine A administration may be due to an increase in osteoclast number and decrease in bone formation at periodontal sites (4–6). However, cyclosporine A has profound effects on the immune system, affecting the whole cytokine network established in periodontal disease. This might result in indirect effects on the nuclear factor-B ligand (RANKL)–osteoprotegerin (OPG) system (7). In support of this hypothesis, it has been shown, in both humans and rats, that cyclosporine A stimulates gene expression of interleukin (IL)-1, IL-6, prostaglandin E_2 (PGE_2) and tumor necrosis factor α (TNF-α), all cytokines known to be involved in bone resorption (8,9). Nitric oxide (NO) produced endogenously by NO donors exerts potent

biphasic actions that profoundly affect the recruitment, proliferation, differentiation activity and/or survival of osteoclasts and osteoblasts, their precursors and other cells within bone (10,11). The relationship between endogenous NO and cyclosporine A-induced alveolar bone loss has not been clearly defined, and the available information regarding NO production in oral tissues is either limited or controversial. However, the presence of inflammatory stimuli, capable of inducing NO production by the inflammatory cells, in the microenviroment of periodontitis, seems to be an important factor in the development of periodontal disease; however, data are still not conclusive (12).

Statins are potent inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR), the proximal and rate-limiting enzyme in the mevalonate pathway (13,14). Inhibition of HMG-CoAR prevents the production of cholesterol (hence the effective use of statins for the treatment of hypercholesterolemia), but also prevents the synthesis of isoprenoid lipids necessary for the prenylation of small guanosine triphosphatases (GTPases), critical signaling molecules that require the addition of an isoprenoid lipid tail to direct them to cell membranes (15). This diminished signaling by GTPases may affect cytokine expression and, coupled with the inhibition of the mevalonate pathway, may result in inhibition of osteoclast differentiation or activation (16,17). This beneficial effect of the statins on bone tissue has been demonstrated by their protective effects against the impact of periodontitis on attachment apparatus and alveolar bone (9), as well as by the promotion of bone formation in the extraction socket (18).

Mundy and colleagues (19) showed that simvastatin can induce the expression of bone morphogenetic protein 2, a member of the transforming growth factor β superfamily and a key regulator of bone morphogenesis. Statins can also stimulate the expression of bone anabolic factors, such as vascular endothelial growth factor (VEGF), and promote osteoblast differentiation and mineralization in MC3T3 cells (20,21). In addition, previous studies have demonstrated that statins inhibit the release of IL-1, IL-6 and TNF-α by upregulating the nuclear receptors, peroxisome proliferator-activated receptor (PPAR-α and PPAR-γ; 22), which may represent a direct antagonist effect to cyclosporine A. Considering the importance of cyclosporine A treatment for a number of medical conditions, and since alveolar bone loss is an important negative side-effect of this drug, we evaluated whether administration of simvastatin *per se* would minimize cyclosporine A-associated alveolar bone loss in rats. The purposes of this study are twofold: firstly, to assess the effects of simvastatin, associated or not with cyclosporine A, on alveolar bone in the presence or absence of inflammation; and secondly, to gain insight into the biological mechanisms associated with modulation of bone turnover by simvastatin and cyclosporine A.

Material and methods

Animals and surgery

All the experimental protocols were approved by the Araraquara Dental School, UNESP Ethical Committee for Animal Research (CEEA). Experiments were performed in agreement with the Ethical Principles in Animal Research, adopted by the Brazilian College of Animal Experimentation (COBEA).

Eighty male Holtzman rats (*Rattus norvegicus albinos* Holtzman), weighing between 90 and 100 g, were randomly distributed into eight experimental groups comprising 10 animals each. The rats were kept in a room with controlled temperature (21 ± 1°C) and humidity (65–70%) and a 12 h–12 h light–dark cycle. Animals were fed standard rat chow and water *ad libitum*.

To induce periodontitis, four groups of rats were anesthetised ($n = 10$ each) by intramuscular administration of ketamine (Francotar®, Virbac of Brazil Ind. and Com. Ltd, São Paulo, Brazil; 80 mg/kg body weight) and xylazine (Virbaxil®, Virbac of Brazil Ind. and Com. Ltd, São Paulo, Brazil; 20 mg/kg body weight), and a 3.0 silk ligature was placed around each rat's right first molar, as previously described (23). One group was treated with cyclosporine A (Sandimmun®; Novartis Pharma AG, Basileia, Switzerland), subcutaneously daily (10 mg/kg body weight, once a day). According to Wassef *et al*. (24), 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 in the absence of experimentally induced periodontal disease (6). One group received oral daily doses (once a day) of simvastatin (Sinvastatina®; Novartis, São Paulo, Brazil) at 20.0 mg/kg. This method has been used successfully in previous studies in this animal model (25,26). The dose was within the range found to be safe and effective for increasing bone density in rats, whereas literature posology reports include both weekly and daily administrations (26). Another group was treated with both cyclosporine A and simvastatin (cyclosporine A/simvastatin) at the indicated doses (once a day), whereas the control group (ligature) received daily subcutaneous injections of sterile saline (0.9% NaCl). The animals in the remaining four groups ($n = 10$ each) were not submitted to periodontitis, but only treated similarly with cyclosporine A, simvastatin, cyclosporine A/simvastatin and saline at the same doses as the ligatured groups. All animals were weighed weekly. The rats were killed 30 days after commencement of the daily treatments.

Collection of samples

At the end of the experimental period, the animals were anesthetised with 0.08 mL/100 g body weight of ketamine (Francotar®) and 0.04 mL/100 g of xylazine (Virbaxil®), and 5–6 ml of blood from the abdominal descending aorta of each rata were drawn into heparinized capillary tubes. After the blood collection, the animals were killed with an overdose of anesthetic.

Subsequently, gingivomucosal tissues surrounding the right first mandibular molar of five rats from each of the eight

Table 1. Primers and conditions used for semi-quantitative RT-PCR

	Primers (5′–3′)	Genbank accession number	Annealling temperature (°C)	Amplicon (bp)	Number of cycles
GAPDH	CACCATGGAGAAGGCCGGGG (sense) GACGGACACATTGGGGGTAG (antisense)	BC083065	52	418	25
iNOS	ACAACAGGAACCTACCAGCTCA (sense) GATGTTGTAGCGCTGTGTGTCA (antisense)	NM012611	54	651	38

groups were removed for evaluation of IL-1β and PGE_2 levels by enzyme-linked immunosorbent assay (ELISA) and evaluation of inducible nitric oxide synthase (iNOS) gene expression at the mRNA level [semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR); Table 1]. These samples were divided into two aliquots, so that one half was used to harvest total protein and the other half was used to harvest total RNA. The remaining five rats in each group had their mandibles removed, trimmed down to include only the first molars and surrounding periodontal tissues and processed for histological/stereometric evaluations.

Serum biochemical analyses

Ionized calcium (Ca^{2+}) — The Ca^{2+} was measured by O-cresolphthalein complexone colorimetric method using and ICA-1 ionized calcium analyzer (Radiometer Company, Copenhagen, Denmark).

Phosphorus — Phosphorous was measured colorimetrically (Kit Phosphorus; Labtest Diagnostica, Lagoa Santa, Brazil). In brief, phosphate ion reacts with the molybdate ion in acid medium, forming a yellow complex that, following the action of an alkaline buffer, is reduced to blue-molybdenum, which is detected by measuring the absorbance at 712 nm using a Technicon SMA-24 spectrophotometer (Technicon, Domont, France). The results are expressed in milligrams per decilitre.

Alkaline phosphatase (ALP) — 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). The units (U/L) of enzyme activity in the experimental sample were calculated from this standard of Bayer units.

Analyses of gene expression on local tissues

Enzyme-linked immunosorbent assays — Total proteins were extracted from the gingivomucosal tissue samples using a detergent-based extraction buffer (tissue protein extraction reagent, T-PER; Pierce, Rockford, IL, USA) containing a protease inhibitor cocktail (protein stabilizing cocktail; Santa Cruz Biotechnology, Santa Cruz, CA, USA), according to the manufacturer's instructions (Pierce Biotechnology). The tissue samples were macerated in the buffer (50 μL/mg of tissue) and centrifuged for 5 min at 2000 ***g*** at 4°C. The concentrated supernatants were quantified using a Bradford protein quantification system (Bio-Rad, Hercules, CA, USA), and the total protein concentration in each sample was used to normalize the results for cytokine gene expression obtained by the ELISAs.

Expression levels of IL-1β and PGE_2 in these samples were determined using commercially available ELISAs [rat IL-1β, Biosource Inc. (San Jose, CA, USA); and rat PGE_2, Assay designs – Correlate – EIA^m] performed according to the supplier's instructions.

Semi-quantitative RT-PCR — Total RNA was extracted from gingival tissue samples using TRIzol™ reagent (Invitrogen, São Paulo, Brazil), according to the manufacturer's instructions. The quantity and purity of total RNA were determined on a Biomate 3 (Thermo Electron Corp., Waltham, Ma, USA) spectrophotometer by the 260 nm and 260 nm/280 nm ratio readings, respectively. Complementary DNA was synthesized by reverse transcription of 800 ng of total RNA using 2.5 μM Oligo(dT) 12–18 primers and 1.25 U/μL Moloney murine leukemia virus reverse transcriptase in the presence of 3 mM $MgCl_2$, 2 mM dNTPs and 0.8 U/μL of RNAse inhibitor, according to the manufacturer's protocol (Improm II; Promega Corp., Santo Agostinho, Brazil). The PCR reaction was performed in a thermocycler (MyCycler; Bio-Rad Laboratories) using 2 μL of the reverse transcriptase reaction product in a 25 μL total volume PCR mix (GoTaq Flexi; Promega Corp.) that contained: 5.3 μL nuclease free water, 4 μL PCR buffer, 0.25 μL Taq DNA polymerase, 0.8 mM dNTPs, 1.5 mM $MgCl_2$ in the presence of 100 pmol/μL of each gene's primers (50 pmol/μL of sense and antisense primers) for both glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and iNOS and genes. The PCR primer sequences and PCR conditions for GAPDH and iNOS are shown in Table 1. The PCR products were resolved by electrophoresis on 1.5% (w/v) agarose gels containing ethidium bromide (0.5 μg/mL) to visualize the PCR products. The amplified DNA bands were analyzed densitometrically after digital imaging capture (Image Quant 100; GE Healthcare, São Paulo, Brazil), using IMAGE J 1.32j software (National Institutes of Health, USA – http://rsb.info.nih.gov/ij/). The density of the bands corresponding to iNOS mRNA in each sample was normalized to the density of the band representing the housekeeping gene,

GAPDH, and expressed as fold change compared to the unstimulated control.

Stereometric analysis

The hemimandibles of five rats from each of the eight experimental groups 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 approximately 3 months. Serial paraffin sections of 5 μm were obtained in buccal–lingual aspects of the whole first left and right molar and subsequently stained with hematoxylin and eosin. Morphological and stereological studies were carried out in the buccal, lingual and interradicular bone regions (Fig. 1, areas 1, 2 and 3, respectively). Each lower first molar has a mesial–distal diameter of approximately 1 mm, producing approximately 160 sections of 5 μm thickness each.

The following stereometrical parameters were quantified, according to the methods used by Huja *et al*. (27). Nomenclature and abbreviations follow the recommendations of the American Society for Bone and Mineral Research (28). Bone volume/total volume [(BV/TV; %) = bone hits/total hits × 400], which represents bone volume (BV; mm^3) per total tissue volume (TV; mm^3); osteoblast surface ($N.Ob/BS/mm^2$) and osteoclast surface ($N.Oc/BS/mm^2$) values indicate the number of osteoblasts and osteoclasts per bone surface, respectively. Measurements were performed on an optical microscope (Carl-Zeiss, São Paulo, Brazil) at a magnification of ×400. The distance between the selected sections was 50 μm. When counting cells, osteoclasts were identified as large multinucleated cells found next to excavated surfaces of alveolar bone (29). Osteoblasts were identified by a typical cuboidal morphology, smaller in size than osteoclasts, with a rounded nucleus and usually found in a single layer, adherent to bone surfaces (30; Fig. 1).

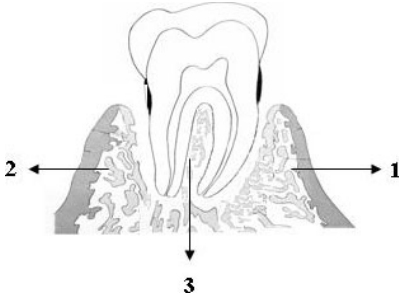

Fig. 1. Schematic illustration showing the regions where volumetric densities of the alveolar bone were measured. 1, buccal bone region; 2, lingual bone region; and 3, interradicular bone region.

Statistical analysis

Measurements were expressed as means and standard deviations of the data collected from all the animals in each group ($n = 5$ for each experiment). One-way ANOVA was used for evaluation of the effect of treatment groups on the variable. Whenever the factor influenced the results, Tukey's *post hoc* test was used to determine pairwise differences between groups. Significance level was set to 5% ($p < 0.05$).

Results

Cyclosporine A and simvastatin modulate serum Ca^{2+} concentration, ALP activity and phosphorous concentration

To determine whether cyclosporine A or simvastatin, administered concurrently or alone, altered the biochemical parameters of bone formation and bone resorption in the serum of rats submitted or not to periodontitis, Ca^{2+} concentration, ALP activity and phosphorous concentration were determined in the sera. As seen in Fig. 2A, cyclosporine A treatment resulted in small but significant decreases in serum Ca^{2+} concentrations ($p < 0.05$), whereas treatment with simvastatin alone induced an accentuated increase in Ca^{2+} concentrations ($p < 0.05$). Interestingly, the treatment with simvastatin, concurrently with cyclosporine A, induced a modest and non-statistically significant increase in Ca^{2+} concentrations. These trends in Ca^{2+} were not altered by the induction of periodontal disease. Phosphorus concentration was significantly decreased by both cyclosporine A and simvastatin in animals not subjected to experimental periodontal disease, with no additive effects resulting from concomitant administration. The induction of periodontal disease resulted in lower serum phosphorus levels in comparison with healthy animals, and treatment only resulted in further significant decreases in these levels (Fig. 2B). In healthy animals, alkaline phosphatase activity was significantly decreased by cyclosporine A, both alone and in association with simvastatin, suggesting an impairment of osteoblast differentiation. Alkaline phosphatase activity in experimentally induced periodontal disease was lower than in healthy control animals and, similarly to healthy control rats, cyclosporine A treatment resulted in a further decrease in ALP. In contrast to the findings in healthy animals, simvastatin treatment in the presence of periodontal disease had no effect on serum ALP levels by itself; however, it did block the cyclosporine A-mediated decrease of ALP (Fig. 2C).

Cyclosporine A decreases IL-1β and PGE_2 expression in periodontal tissues, but has opposite effects on healthy and inflamed tissues

The levels of IL-1β and PGE_2 were determined by ELISA. As shown in Fig. 3A,B, both IL-1β and PGE_2 concentrations were significantly increased by cyclosporine A treatment in non-inflamed periodontal tissues, whereas in the presence of inflammation, cyclosporine A treatment had the opposite effect of decreasing the expression levels of both cytokines. Simvastatin treatment reduced expression of both IL-1β and PGE_2 in the presence and absence of inflammation. Interestingly, in the absence of inflammation, simvastatin treatment completely abrogated the cyclosporine A-induced increase in IL-1β, whereas PGE_2 levels were only marginally affected by concomitant cyclosporine A/simvastatin treatment. Also, in the presence of inflammation, simvastatin and cyclosporine A presented additive effects only on the reduction of IL-1β.

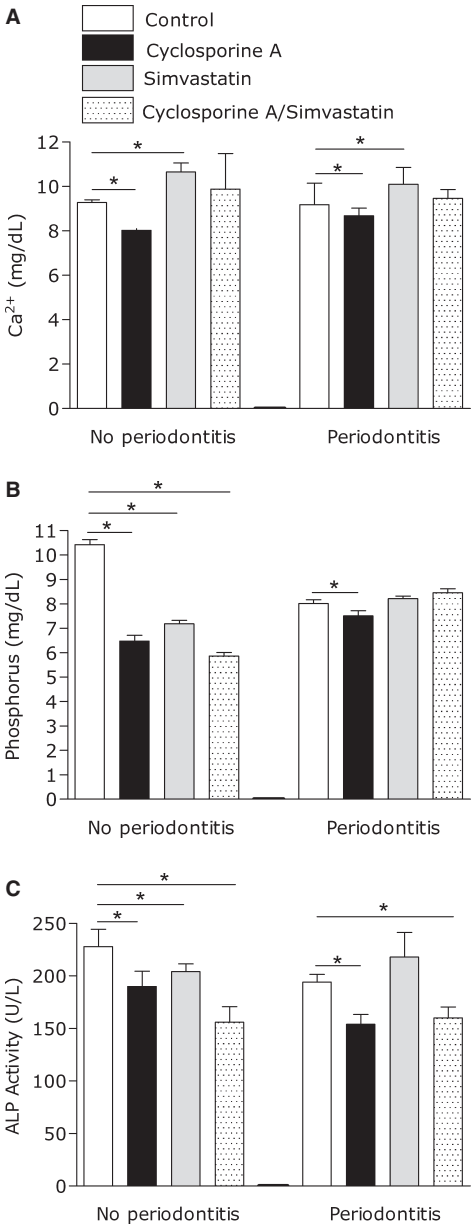

Fig. 2. Serum concentrations of bone turnover markers measured in rats following the different treatments: cyclosporine A (10 mg/kg s.c.), simvastatin (20.0 mg/kg p.o.) or cyclosporine A/simvastatin or 0.9% NaCl (s.c.), with or without ligature, for 30 days. Ionized calcium levels and phosphorus levels were reduced by cyclosporine A treatment, but increased by simvastatin or cyclosporine A/simvastatin treatment (A,B). Alkaline phosphatase activity decreased in cyclosporine A- and cyclosporine A/simvastatin-treated rats, but increased after simvastatin alone. All values are expressed as means ± SEM (n = 10 animals per group). *$p < 0.05$.

Cyclosporine A and simvastin have similar effects on iNOS mRNA regulation

The expression of iNOS mRNA was analyzed by semi-quantitative RT-PCR, as shown in Fig. 4. There was a significant increase in the expression of iNOS mRNA in the non-inflamed tissues of cyclosporine A-treated rats ($p < 0.05$). The effects of simvastatin treatment in the absence of inflammation were similar to those of cyclosporine A. Induction of inflammation by the placement of ligatures resulted in a significant increase in iNOS mRNA levels, in comparison to untreated healthy tissues, and treatment with cyclosporine A and simvastatin, either singly or together, resulted in inhibition of iNOS mRNA expression.

Stereometric analysis

Mean (BV/TV; %), N.Ob/BS and N.Oc/BS values, measured in the buccal, interradicular and lingual regions of the mandibular first molar are shown in Fig. 5A–C. Figure 6 shows typical micrographs of the decalcified transverse sections of the interradicular region of the first mandibular molar from rats without ligatures [control animal (A), cyclosporine A treated (B), simvastatin treated (C) and cyclosporine A/simvastatin (D)] and rats with ligatures [control animal (E), cyclosporine A treated (F), simvastatin treated (G) and cyclosporine A/simvastatin (H)] at the end of the 30 day treatment period. Histological examination of decalcified sections revealed that cyclosporine A-treated rats (without ligature) presented with alveolar bone loss, characterized by the eroded surface of trabecular bone and increased bone marrow. A decrease in BV/TV was associated with a decrease in the number of osteoblasts per unit surface area (N.Ob/BS) and a significant increase in the number of osteoclasts per unit surface area (N.Oc/BS). The cyclosporine A accentuated alveolar bone loss in rats with ligature. In contrast, histological examination revealed no differences in alveolar bone, BV/TV, N.Ob/BS or N.Oc/BS measurements between simvastatin-treated and respective control rats (in both rats without ligature and rats with ligature), suggesting that simvastatin treatment did not have a measurable effect on alveolar bone turnover. However, concomitant administration of cyclosporine A/simvastatin demonstrated that simvastatin was able to completely block the deleterious effects of cyclosporine A on alveolar bone, with the exception of osteoclast count in non-inflamed tissues, which was not completely abrogated by simvastatin (Fig. 5C).

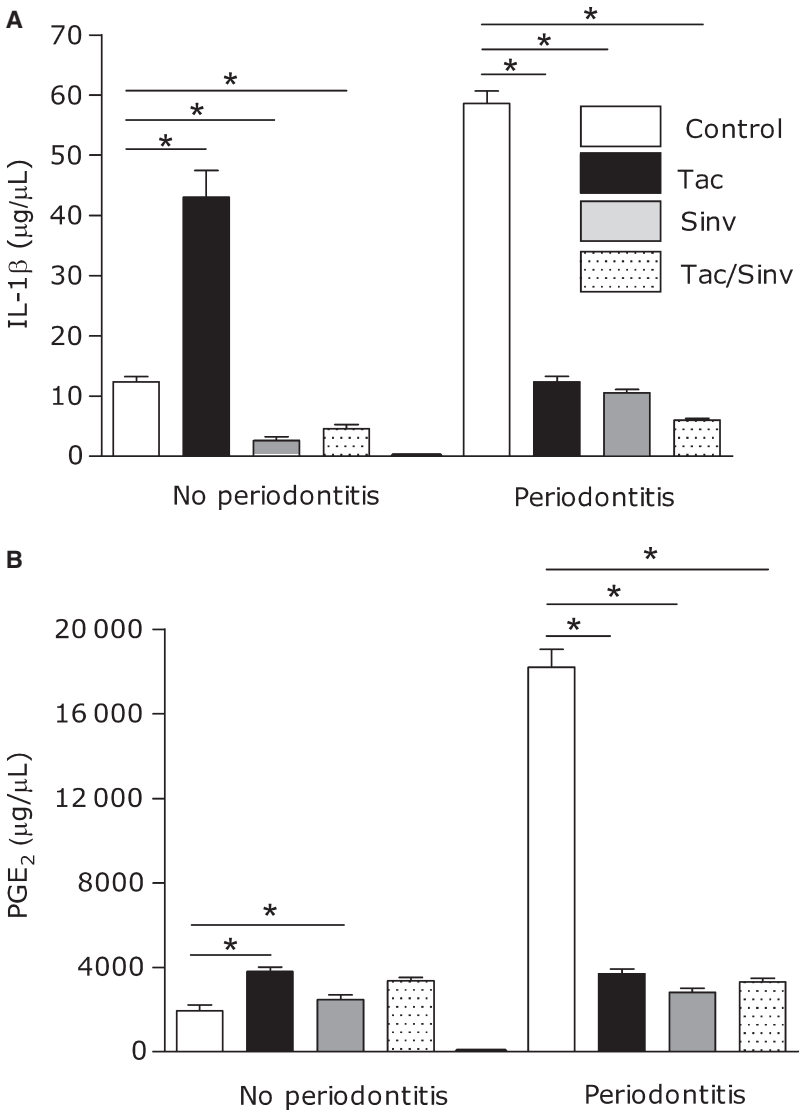

Fig. 3. Gingivomucosal concentration of IL-1β (A) and PGE_2 (B) measured in rats following the different treatments. All values are expressed as means + SEM (n = 10 animals per group). *$p < 0.05$.

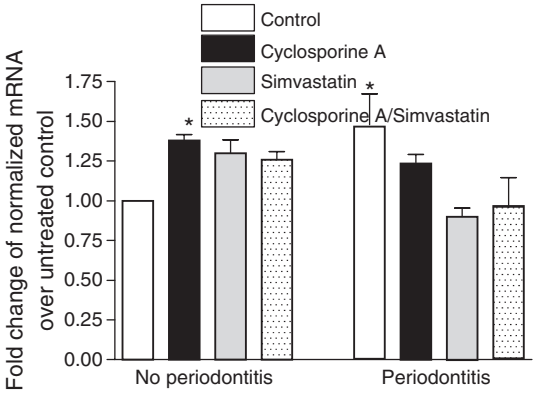

Fig. 4. Gingivomucosal concentration of iNOS mRNA, measured in rats following the different treatments. All values are expressed as means + SEM (n = 10 animals per group). *$p < 0.05$.

Discussion

Recently, we suggested that the alveolar bone loss associated with cyclosporine A administration might be minimized by concomitant treatment with alendronate (31). However, severe side-effects associated with alendronate use have been reported owing to the impairment of vascularization. The biological effects of statins on bone metabolism were first reported in 1999, when Mundy *et al.* (19) found that statins were potent stimulators of bone formation *in vitro*. In fact, results of many studies strongly suggest that statins have a beneficial effect on bone health (32). We could not find any studies regarding the effects of simvastatin on cyclosporine A-induced alveolar bone loss associated, or not, with experimentally induced periodontitis. Therefore, in the present study, we used an established and well-characterized animal model to evaluate, firstly, the role of simvastatin *per se* in the prevention of cyclosporine A-induced alveolar bone loss and on the presence and absence of inflammation and, secondly, the effects of simvastatin and cyclosporine A on biochemical parameters of bone turnover and on the expression of cytokines that are relevant for bone resorption. In this study, histological observations confirmed that administration of immunosuppressive doses of cyclosporine A for 30 days caused alveolar bone loss, in agreement with previous reports (4,5). Our findings also confirmed that treatment with cyclosporine A accentuated alveolar bone loss in rats with ligature. In addition, we showed that administration of simvastatin counteracted the deleterious effects of cyclosporine A on bone turnover in the absence of inflammation. These results are compatible with those reported by Ohno *et al.* (33), who showed that treatment with cerivastatin (a synthetic statin) improves cyclosporine A-induced high-turnover osteopenia in transplanted bone, mainly through the inhibition of bone resorption. In the present study, the histological observations were confirmed by stereometry, and we can thus suggest that the

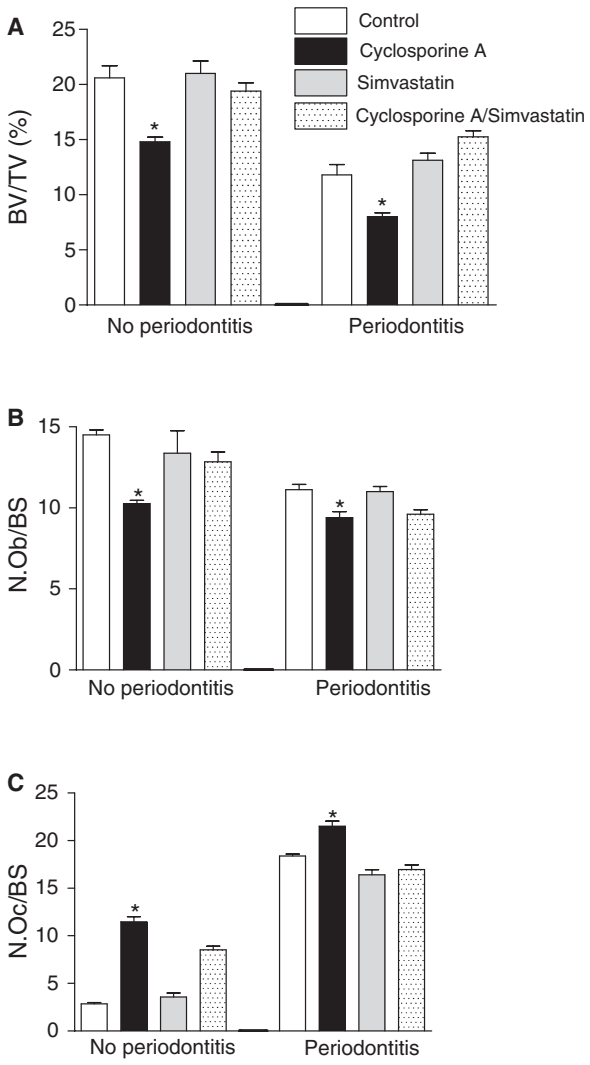

Fig. 5. Average stereometric measurements from the buccal, interradicular and lingual regions of the mandibular first molar obtained from rats following the different treatments. (A) BV/TV; %. (B) Number of osteoblasts per bone surface (N.Ob/BS). (C) Number of osteoclasts per bone surface (N.Oc/BS). All values are expressed as means + SEM ($n = 10$ animals per group). *$p < 0.05$ vs. the other groups within the same experiment (i.e. with or without ligature).

decrease in BV/TV seen during cyclosporine A treatment was the result of a lower trabecular number (Tb.N) and thickness (Tb.Th) and higher trabecular separation (Tb.Sp; data not shown). Our results also show that the decrease in BV/TV resulting from cyclosporine A treatment was associated with a significant decrease in the number of osteoblasts (N.Ob/BS) and an increase in osteoclasts (N.Oc/BS), which was significantly affected by simvastatin in both non-inflamed and inflamed periodontal tissues. The serum biochemical markers corroborated the histological findings of cyclosporine A-mediated impairment of bone turnover, as indicated by a small but statistically significant decrease in both Ca^{2+} and phosphorus concentrations. Even though the serum Ca^{2+} concentrations observed confirmed our previous findings (5,6), we cannot rule out the possibility [suggested by Mason *et al.*(34) and Ryffel *et al.*(35)] that this could be the result of a non-specific effect of cyclosporine A on renal excretion of Ca^{2+}. Other studies performed in rats treated with comparable immunosuppressive doses of cyclosporine A have shown a severe bone loss without any changes in serum ionized calcium (31,36). It should be pointed out, however, that simvastatin administration alone or in association with cyclosporine A resulted in a small increase in both Ca^{2+} and phosphorus concentrations in the presence of experimentally induced periodontal disease. In contrast, in rats with healthy periodontium, simvastatin treatment had opposite effects on Ca^{2+} and phosphorus serum levels. Collectively, these results suggest that simvastatin therapy had a beneficial effect on bone turnover. We are not aware of any study that directly evaluates the association between serum phosphorus concentration and alveolar bone loss in rats with or without experimentally induced periodontal disease.

In accordance with our recent studies (5,6), cyclosporine A administration resulted in significant decreases in ALP activity, in the presence or absence of experimentally induced periodontal disease, suggesting a probable action of this drug on osteoblast maturation and, consequently, bone resorption. Simvastatin treatment also resulted in a decrease of ALP in healthy animals, whereas the opposite effect was observed in the presence of periodontal inflammation. However, simvastatin had no counteracting effect on the cyclosporine A-mediated decrease of ALP activity in healthy or periodontally disease animals. Maeda *et al.* (20) suggested that simvastatin enhances ALP activity and promotes osteoblast differentiation and mineralization. Interestingly, when cyclosporine A and simvastatin were administered concurrently, an additive effect on the decrease of ALP activity was observed. In this case, the action of cyclosporine A could surpass that of simvastatin on ALP activity.

In contrast, it has been suggested that the osteopenic actions of cyclosporine A are not due to its direct effects on bone cells, but occur via T-cell modulation that interferes in their cytokine expression, in turn affecting both osteoblasts and osteoclasts in the microenvironment, and ultimately influencing bone remodeling (37). For example, it has recently

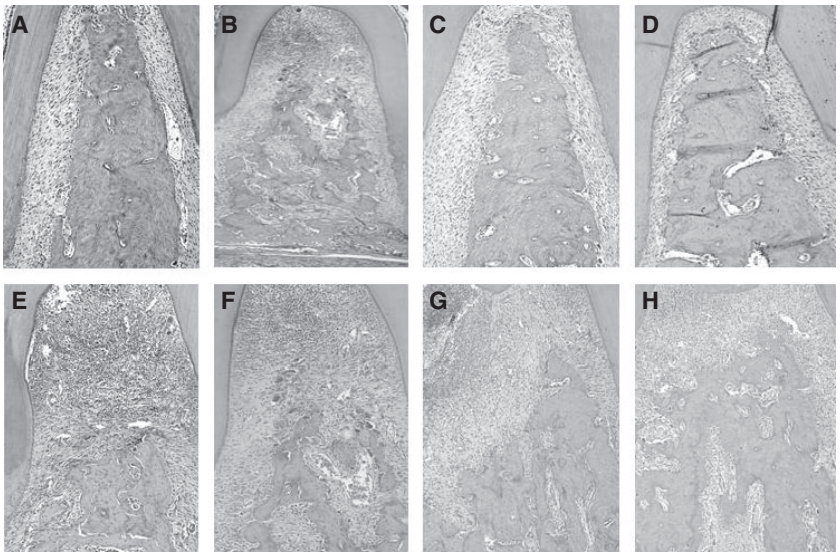

Fig. 6. Decalcified transverse sections of the interradicular region of mandibular first molars obtained from rats without (A,B,C and D) or with (E,F,G and H) periodontitis following the different treatments. (A) and (E) control (untreated); (B) and (F) cyclosporin A (10 mg/kg/day, s.c. 30 days); (C) and (G) simvastatin (20 mg/kg/day, oral, 30 days); (D) and (H) cyclosporin A and simvastatin. (Hematoxylin and eosin; bar: 25 μm) (stained with hematoxilin and eosin).

been demonstrated that cyclosporine A inhibits osteoclast formation by inhibiting calcineurin and, subsequently, the expression of the nuclear factor of activated T-cells c1 (NFATc1), a transcription factor involved in osteoclast differentiation and function (38). It is interesting that the immunosuppressive drug, cyclosporine A, produced increases in IL-1β and PGE_2 protein and iNOS mRNA in non-inflamed tissues, and a similar regulation of these genes has also been observed in experimentally induced inflammation. However, in the presence of inflammation, cyclosporine A exerted its immunosuppressive effects by decreasing the expression levels of these genes. Considering the complex nature of the cytokine network established in inflamed periodontal tissues, it is possible that cytokines that are upregulated by the inflammatory response have redundant biological effects that may compensate the effects associated with the cytokines whose expressions are decreased by cyclosporine A, such as IL-1β or PGE_2, shown in this study. This complexity of the cytokine network during inflammation could also explain the contradictory effects of cyclosporine A on cytokine gene expression in the presence and absence of inflammation.

In the present study, we showed that cyclosporine A treatment resulted in increased concentrations of IL-1β, PGE_2 and iNOS mRNA expression in the absence of inflammation. These cytokines are considered to play a role in bone resorption, probably through the stimulation of osteoclast proliferation and differentiation, leading to bone loss (39). Simvastatin treatment resulted in a significant decrease in constitutive and cyclosporine A-induced expression of IL-1β and PGE_2 in both normal and inflamed periodontal tissues. The increase in PGE_2 levels with cyclosporine A treatment in normal tissues, as well as with inflammation induced by ligature placement, could induce a potentiation of alveolar bone resorption through osteoblast-mediated osteoclast differentiation. In fact, *in vitro* experiments suggest that PGE_2 is primarily produced by osteoblasts. More importantly, PGE_2 acts in an autocrine fashion on osteoblasts to form osteoclasts, leading to bone resorption (39). However, since cyclosporine A decreased the expression of IL-1β, PGE_2 and iNOS, other cytokines may play a role in the aggravation of inflammation-associated alveolar bone loss by cyclosporine A. Nitric oxide is a neuronal chemical messenger of particular interest owing to its chemical identity, wide distribution and broad spectrum of effects (40). Nitric oxide is synthesized from L-arginine by the enzyme, nitric oxide synthase (NOS). Currently, at least three main isoforms of NOS are known to exist, which are encoded by different genes and have different molecular weights. Two of them are constitutive (neuronal and endothelial) and one is inducible (iNOS; 41). In fact, the inducible nitric oxide synthase is thought to have an important role in the pathogenesis of inflammatory periodontitis, as it does in other inflammatory diseases, contributing to the amplification and progression of the inflammatory infiltrate, modulating the activity of several cells and stimulating apical migration of the junctional epithelium, destruction of collagen fibers and bone resorption (42). In the rodent model, NO is known to upregulate matrix metalloproteinase activity, potentiating matrix degradation (43). The presence of inflammatory stimuli, capable of inducing NO production by the inflammatory cells, in the microenvironment of periodontitis is also well established. The detection of high amounts of the NO precursors, L-arginine and L-citrullin, in inflamed gingival tissue *in vivo* (44), and iNOS expression in macrophages, lymphocytes and polymorphonuclear neutrophils in experimentally induced periodontitis in rats, as well as in macrophages, endothelial cells and polymorphonuclear neutrophils in human periodontitis, suggests the production and participation of NO in periodontitis (12,44). Simvastatin treatment potentiated the suppressive effects of cyclosporine A on iNOS mRNA levels in inflamed tissues, whereas a slight increase in iNOS mRNA levels was observed with simvastatin treatment, similar to the effects of cyclosporine A alone. Interestingly, simvastatin treatment always

had an inhibitory effect on IL-1β and PGE_2 expression. It is important to acknowledge that iNOS expression was studied only at the mRNA level in this study. In fact, Kasten *et al*. and Batista *et al*. (10,45) have shown that osteoclasts and related osteoclast-like cells express iNOS and release NO in a regulated manner. Nitric oxide, produced endogenously or supplied by NO donors, exerts potent biphasic actions that profoundly affect the recruitment, proliferation, differentiation, activity and/or survival of osteclasts and osteoblasts, their precursors and other cells within bone (45,46).

In summary, the present study showed that treatment of rats with cyclosporine A results in induction of alveolar bone loss in non-inflamed periodontal tissues and in aggravation of alveolar bone loss in inflamed periodontal tissues. This effect was associated with an increase in osteoclast density, contradictory levels of IL-1β and PGE_2 protein and iNOS mRNA levels in the periodontal tissues, and altered biochemical parameters. In addition, treatment with simvastatin decreased osteoclast density and counteracted the effects of cyclosporine A on cytokine gene expression, consequently reducing the deleterious effects of cyclosporine A on alveolar bone loss associated or not with experimentally induced periodontal disease. As such, and within the limitations of this study, we conclude that the treatment with simvastatin is a beneficial therapeutic option that favours the normalization of cyclosporine A-induced alterations in bone metabolism. Additional studies are being designed and conducted in order to faciliate understanding of the biological mechanisms for the differential actions of cyclosporine A and simvastatin in the presence and absence of inflammation.

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