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Effect of cyclosporin A on alveolar bone homeostasis in a rat periodontitis model

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Objectives: The administration of cyclosporin A has been associated with significant bone loss and increased bone remodeling. The present investigation was designed to evaluate the effects of cyclosporin A on alveolar bone of rats subjected to experimental periodontitis, using serum, stereometric and radiographic analysis.

Methods: Twenty-four rats were divided into one of the following groups with six animals each: group I, control rats; group II, in which the animals received a cotton ligature around the lower first molars; group III, in which the rats received a cotton ligature around the lower first molars and were treated with 10 mg/(kg body weight day) of cyclosporin A; group IV, in which the rats were treated with 10 mg/(kg body weight day) of cyclosporin A. At the end of experimental period, at 30 days, animals were killed and the serum calcium and alkaline phosphatase levels were measured in all groups. The distance from the alveolar bone crest to the cemento–enamel junction was measured radiographically for each mesial surface of the lower first molars of each rat. After histological processing, the stereological parameters: volume densities of multinucleated osteoclasts (V_o), alveolar bone (V_b), marrow (V_m), and relation of eroded surface/bone surface (Es/Bs) were assessed at the mesial region of the alveolar bone.

Results: Significant decreases in serum calcium were observed in those groups that received cyclosporin A therapy. No significant changes in serum alkaline phosphatase were observed. The therapy with cyclosporin A combined with the ligature placement decreased the V_b and increased the V_o, V_m and Es/Bs at the mesial surface of lower first molars. On the other hand, the radiographic data showed that cyclosporin A therapy diminished the alveolar bone loss at the mesial surface of the lower first molars.

Conclusions: Therefore, within the limits of this study, we suggest that cyclosporin A at immunosuppressive levels can bring about an imbalance in the alveolar bone homeostasis in rats. However, in the presence of inflammatory stimulation, the inhibition of the immune system by cyclosporin A may decrease the initial periodontal breakdown.

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The use of cyclosporin-A to prevent rejection of allograft tissue has stimulated the field of transplantation biology (1). Cyclosporin A prevents activation of CD4 lymphocytes mainly by inhibiting the transcription of interleukin-2 gene through interaction with cytoplasmic receptors in these cells (2). However, the use of this drug is associated with significant adverse reactions, including metabolic disturbances (3), nephropathy (4), hypertension (5), gingival overgrowth (6, 7), and osteopenia (8, 9).

Some *in vitro* studies have indicated that cyclosporin A decreases bone

resorption by specifically inhibiting T lymphocyte-derived messengers such as interleukins (IL-1α, IL-2, IL-3, and IL-4), gamma-interferon, and tumor necrosis factor-alpha (10), which are potent bone degrading agents (8). However, some other *in vitro* studies indicate that cyclosporin A can enhance the release of some arachidonate metabolites, in particular prostaglandin E2 (PGE2) and thromboxane (11–14). In human gingival fibroblasts culture, cyclosporin A treatment has been shown to result in enhanced excretion of PGE2 (15), a cyclooxygenase-2 (COX-2) metabolite, mediator of osteoclastic bone resorption (16).

There is also some controversy regarding the *in vivo* effects of cyclosporin A on bone metabolism. Some studies demonstrated that cyclosporin A decreased bone resorption and increased bone formation in rats (17), and in arthritis-related osteopenia cyclosporin A normalized the bone remodeling (18). However, a severe osteopenia responsible for osteoporotic fractures in transplantation recipients has been described by some authors (19–21). Furthermore, an increased osteoclasia and decreased bone formation at periodontal sites have been observed in the alveolar bone of rats treated with cyclosporin A (22–24). To further explore the effect of cyclosporin A on bone resorption, this present study was designed to investigate the effects of cyclosporin A administered at immunosuppressive levels on alveolar bone of rats subjected or not to experimental periodontal disease, using radiographic, stereometric, and blood analysis.

Material and methods

Animals

Twenty-four Holtzman rats (*Norvergicus albinus*) weighing an average of 100 g were randomly distributed into four groups of six animals each. All the rats were housed under similar conditions and maintained on diet and water *ad libitum*.

One group was used as controls (group I) and received subcutaneous injection of saline solution during 30 days.

Protocol of experimental periodontal disease

Animals from groups II and III were subjected to experimental periodontitis. After general anesthesia with intramuscular injection of 0.08 ml/100 g body weight of ketamine (Francotar®, Virbac do Brazil Ind. e Com. Ltda, São Paulo, Brazil), a cotton thread ligature was surgically placed around the cervix of the mandibular first molars on both sides (right and left). The ligature was knotted on the vestibular side, so that it remained subgingivally in the palatinal side (25, 26).

Cyclosporin A

Animals from groups III and IV received cyclosporin A therapy. Cyclosporin A (Sandimmun, Sandoz, Basel, Switzerland) was injected subcutaneously, in a daily dose of 10 mg/kg body weight (27) during 30 days. This treatment gives estimated peak and through levels of cyclosporin A of 1000 ng/ml and 750 ng/ml, respectively. The rats were weighed weekly and monitored for abnormal appearance of coat, abnormal level of activity, or respiratory distress.

Serum analysis

At the end of the experimental period or at 30 days, these animals were anesthetized using ketamine anesthesia and 4–5 ml of blood was obtained by direct cardiac puncture. All blood samples were analyzed in a Technicon SMA-24 (Francotar®, Virbac do Brazil Ind. e Com. Ltda, São Paulo, Brazil). Serum calcium and total serum alkaline phosphatase levels were obtained from each animal. After the cardiac puncture, the rats were killed.

Radiographic analysis

The mandibles were carefully removed, and soaked in 10% formalin for 48 h. Then, in order to measure the amount of bone, standardized digital radiographs were obtained with the use of a computerized imaging system, CDR^R (Francotar®) (26). Electronic sensors were exposed at 65 KV and 10 mA. The source-to-film distance was always set at 50 cm. The amount of alveolar bone loss, expressed by the distance from the alveolar bone crest to the cemento–enamel junction, was measured (in mm) three times, in different days and by the same examiner, for each mesial surface of the mandibular first molars on each radiograph (26). Following radiographic examination, the mandibles were decalcified in solution of Morse (50 ml of 50% formic acid and 50 ml of 20% sodium citrate). Serial paraffin sections of 5 μm were made on the mesio-distal, and stained with haematoxylin and eosin. Stereometrical studies were made on the mesial region.

Stereometry

Volume densities of multinucleated osteoclasts (V_o), alveolar bone (V_b), marrow (V_m) and relation of eroded surface/bone surface (Es/Bs, %) were estimated according to the principles established by Dellesse (28), which were applied to histology by Weibel (29). The count was performed with the help of a Zeiss microscope, used at a magnification of ×200. A square lattice of 25 points was projected into the microscope ocular, with the use of a microvid system that connected the microscope to a computer. For each animal, 20 sections were used, and 25 points were counted in each section. V_o, V_b, V_m and Es/Bs were expressed as percentage of the total points counts.

Statistical analysis

Summary statistics included mean ± standard deviation (SD). Comparisons between groups regarding the mean serum calcium and total serum alkaline phosphatase levels, as well as the stereological data, were made using analysis of variance. The hypothesis that there were no differences in bone loss rate among treatment groups was tested by an intergroup analysis using one-way analysis of variance (ANOVA). Pairwise multiple comparisons were

carried out by the Tuckey test in the cases where ANOVA test showed significant differences. Significance level was always set at 95%.

Results

Serum data

Table 1 shows the serum calcium and alkaline phosphatase levels of the rats treated with cyclosporin A and/or subjected to the experimental periodontitis. In the control group and in the experimental periodontitis group II, the serum calcium and alkaline phosphatase levels were 10.6 ± 0.3 mg/dL, 843 ± 76 U/dL and 10.1 ± 0.2 mg/dL, 836 ± 81 U/dL, respectively. In the groups treated with cyclosporin A, groups III and IV, the serum calcium levels decreased significantly when compared with the groups not treated with cyclosporin A ($p < 0.05$). A decrease in serum alkaline phosphatase levels was also observed in the groups treated with cyclosporin A; however, these values were not statistically different from those of the control group.

Table 1. Effect of cyclosporin A and/or experimental periodontitis on serum calcium and alkaline phosphatase

Groups	Calcium (mg/dl)	Alkaline phosphatase (U/dl)
I	10.6 ± 0.3	843 ± 76
II	10.1 ± 0.2	836 ± 81
III	9.0 ± 0.2*	780 ± 91
IV	9.3 ± 0.3*	760 ± 76

Data are represented as mean ± SEM when $n = 6$.
*$p < 0.05$, statistical significance vs. control rats.

Table 2. Mean and standard deviation (SD) of the alveolar bone loss (mm) measured under radiographic analysis as the distance between cemento–enamel junction and the alveolar bone crest

Groups	Alveolar bone loss mean ± SD (mm)
I	1.05 ± 0.08
II	1.85 ± 0.16*#
III	1.66 ± 0.11*†
IV	1.37 ± 0.08*‡

*Significant difference from control.
#Significant difference ($p < 0.05$) from groups treated with cyclosporin A (groups III and IV).
†Significant difference ($p < 0.05$) from groups subjected to the experimental periodontitis alone (group II) and treated with cyclosporin A alone (group IV).
‡Significant difference ($p < 0.05$) from groups subjected to the experimental periodontitis (groups II and III).

Effects of cyclosporin A on radiographic alveolar bone height

The distance between the cemento–enamel junction and the alveolar bone crest was significantly affected by cyclosporin A therapy (Table 2). Significantly increased bone loss was observed in the cyclosporin A groups (III and IV) compared with control group.

However, ligature placement alone during 30 days (group II) lead to a significantly higher bone loss ($p < 0.05$) when compared with cyclosporin A therapy alone or combined with experimental periodontitis (groups IV and III, respectively).

Stereometry

In the control group, the volume densities of osteoclast (V_o) at the mesial face was 2.6 ± 2.8% (Table 3). The volume densities of osteoclasts increased in the rats submitted to the experimental periodontitis (group II: 18.1 ± 8.1%), and this increase was more pronounced when experimental periodontitis was associated with cyclosporin A therapy (group III: 20.6 ± 3.2%).

Table 3. Volumetric densities of osteoclast (V_o), bone (V_b) and marrow (V_m), and eroded surface/bone surface (Es/Bs) of the alveolar bone of the lower first right molar of control rats, and rats treated with cyclosporin A and/or experimental periodontal disease

Groups	V_b (%)	V_m (%)	V_o (%)	Es/Bs (%)
I	0.72 ± 0.02	0.28 ± 0.03	2.6 ± 2.8	13.1 ± 11.9
II	0.56 ± 0.01*	0.44 ± 0.01	18.1 ± 8.1*	30.8 ± 13.7*
III	0.45 ± 0.02*#	0.55 ± 0.02*#	20.6 ± 3.2*#	36.5 ± 6.6*#
IV	0.65 ± 0.01*	0.35 ± 0.01*	15.5 ± 4.3*	25.9 ± 8.3*

All values represent the mean ± SEM.
*Significantly different from control group ($p < 0.05$).
#Significantly different from treatment groups II and IV ($p < 0.05$).

The values for eroded surface/bone surface (Es/Bs), were statistically higher in all experimental groups in comparison to the control group. The highest values were found in the rats subjected to the experimental periodontitis and treated with cyclosporin A (group III: 36.5 ± 6.6). The values for V_b were statistically smaller in all groups treated with cyclosporin A in comparison to the control group ($p < 0.05$). The smallest values for V_b were found in the association of experimental periodontal disease and treatment with cyclosporin A (group III: 0.45 ± 0.02). Opposite results were observed regarding the V_m ($p < 0.05$).

Discussion

This study was designed to evaluate the effects of immunosuppression induced by cyclosporin A on periodontal breakdown of rats subjected to ligature-induced periodontal disease. The experimental model of periodontitis employed in the present study

is characterized by a progressive accumulation of plaque and increasing infiltration of inflammatory cells, which lead to a degradation of the periodontal connective tissues and bone (25, 26). In the present study the period of treatment was based on previous observations (26, 30, 31) which have demonstrated that a severe bone destruction is evident after 30 days of ligature placement in rats.

The experimental period for the treatment with cyclosporin A was also based on a previous study by Schlosberg *et al.* (32) who have showed that the effect of cyclosporin A on bone and mineral metabolism is dependent on the duration of treatment. They observed an increased bone resorption, represented by a significant increase in osteoclast-like cell number, accompanied by a reduction in the bone volume of 79%, within 28 days of cyclosporin A treatment. In agreement with Schlosberg *et al.* (32) and other previous studies (22, 23, 32–36), this present investigation showed that 30 days of cyclosporin A administration, in doses that have been reported to be immunosuppressive in the rat (37), resulted in striking and unique histomorphometric and serum changes in rat bone mineral metabolism at the end of experimental period. The data presented in this study indicate that cyclosporin A at a dose of 10 mg/(kg body weight day) leads to an increase in the volumetric densities of osteoclasts (V_o) and marrow (V_m) and in the relation of eroded surface/bone surface (Es/Bs %). On the other hand, the volume densities of bone (V_b) decreased, indicating bone loss.

However, some *in vitro* studies, in isolated organ or bone cell cultures, showed inhibition of the bone resorption (38–40). According to Movsowitz (33), these differences of the effects of cyclosporin A on bone metabolism between *in vitro* and *in vivo* studies may be due to the fact that the whole animal is more representative of the influence of multiple and complex interactions between cyclosporin A and the immune system compared with very selected and often isolated local factors studied *in vitro* systems.

It is believed that in the normal physiological situation, both bone formation and resorption progress in a balanced, regulated manner with osteoclastic bone resorption preceding new bone formation by osteoblasts (41). The presence of cyclosporin A, however, brings about an imbalance in this dynamic remodeling cycle, with excess resorption far exceeding formation, leading to an ultimate loss of bone (42). The mechanisms by which cyclosporin A induced such osteopenia remain unclear, although some hypotheses have been presented. Buchinsky *et al.* (43), postulated that cyclosporin A may exert its osteopenic effect via the T-cell rather than directly on bone. Cyclosporin A may mediate its osteopenic effect by interfering in the cytokine activity on both osteoclasts and osteoblasts at the bone microenvironment (39, 44) thus influencing bone remodeling (38). Compatible with stereological findings of previous reports (22, 24, 32), an increase in the volume density of osteoclasts was shown in the present study in both groups treated with cyclosporin A.

In a recent report (45), it was showed that cyclosporin A did not inhibit COX-2 expression induced by bacterial challenge in gingival connective tissue cell cultures. In fact, evidence is now available which suggests that cyclosporin A has been shown to induce an up-regulation of the biosynthesis of eicosanoids in monocytes (12), lymphocytes (13), smooth muscle cells (14) and human gingival fibroblasts (15). There is strong evidence suggesting the involvement of eicosanoids in the tissue destruction in periodontal disease (46). It is well documented that prostaglandins of the E series are powerful mediators of osteoclastic bone resorption and can affect both the active mature osteoclasts as well as differentiated osteoclast precursors (16). A previous study by Wondimu *et al.* (15) showed that cyclosporin A enhanced PGE2 formation in human gingival fibroblasts without any further increase in the level of COX-2 mRNA induced by tumor necrosis factor-alpha. According to this author, these findings indicate that the potentiation of PGE2 formation induced by cyclosporin A is not due to an enhanced level or activity of the cyclooxygenase enzyme but rather to an increased level and/or activity of phospholipase A2, the enzyme responsible for the release of arachidonic acid from phospholipids in the cell membrane. Thus, it can be suggested that cyclosporin A may also affect the production of the metabolites of the lipoxygenase pathway, which can also stimulate osteoclastic bone resorption (47). This is especially relevant in the light of the present data showing that in rats with ligature, the administration of cyclosporin A resulted in a more accentuated effect on the number of osteoclasts and on the percentage of eroded surface when compared with rats with ligature that did not receive cyclosporin A.

Despite the evidence of an increased number of osteoclasts and increased resorptive surfaces, the radiographic analysis showed less alveolar bone loss in rats with periodontitis treated with cyclosporin A, as compared with rats with periodontitis alone. It is well established that the association between the immune system and the pathogenesis of periodontitis is apparent (16, 46). Thus, we can suggest that the inhibition of the immune system by cyclosporin A could have minimized the intensity of the inflammatory reaction in the initial phase, induced by the placement of ligature. A possible reason for this observation is that since cyclosporin A is known to inhibit specifically the T cell response (2), which is predominant in early periodontal lesions in rats (48), it might have interfered with the onset of periodontitis, decreasing the total amount of alveolar bone resorption observed at the end of the experimental period.

The biochemical assessment of biomarkers of bone turnover correlated well with the histological findings. Our present experiment showed a significant decrease in serum calcium level, which, according to Mason (49) and Ryffel (50), could be a non-specific effect of cyclosporin A due to an increased excretion by kidney. In contrast with our findings, in other rat experiments, comparable immunosuppressive doses of cyclosporin caused a

severe high turnover osteopenia without changes in ionized calcium, phosphate or PTH levels (33, 34). In accordance with previous studies (44), a decrease (although not significant) in serum alkaline phosphatase level, a marker of osteoblast phenotype (51), was also observed in the present study in cyclosporin A-treated groups, suggesting a modest negative effect of cyclosporin A on bone formation. Assuming bone formation is coupled to bone resorption, these biochemical findings could explain the significant greater number of osteoclasts and percentage of eroded bone surface encountered in rats with experimental periodontitis associated with cyclosporin A treatment in the present study.

Within the limits of this study, we suggest that cyclosporin A at immunosuppressive levels can bring about an imbalance in the alveolar bone homeostasis in rats. However, in the presence of inflammatory stimulation, the inhibition of the immune system by cyclosporin A may decrease the initial periodontal breakdown. Other studies, with shorter and longer periods of evaluation, are necessary in order to clarify the role of this metabolic disturbance on the alveolar bone height.

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