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Effects of long-term cyclosporin therapy on the periodontium of rats

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Background: The treatment of cyclosporin A triggers an early bone loss and gingival overgrowth. There is a lack of studies exploring the effects of long-term cyclosporin A therapy on alveolar bone homeostasis and gingival tissue.

Objective: The purpose of this study was to evaluate the effects of long-term therapy with cyclosporin A on the gingival tissue and on the alveolar bone metabolism in rats.

Materials and methods: Rats were treated for 60, 120, 180 and 240 days with a daily subcutaneous injection of 10 mg/kg body weight of cyclosporin A. At the end of experimental periods, animals were killed and the serum calcium (Ca^{2+}) and alkaline phosphatase levels were measured in all groups. After histological processing, the oral epithelium and the connective tissue, as well as volume densities of alveolar bone (V_b) and multinucleated osteoclasts (V_o), were assessed at the region of the lower first molars.

Results: Significant increases in the serum alkaline phosphatase were observed in those groups that received cyclosporin A therapy. After 60 and 120 days of the treatment with cyclosporin A, evident gingival overgrowth associated with a significant increase of epithelium and connective tissue was observed, as well as a decrease of the densities of bone and an increase of densities of osteoclasts. After 180 and 240 days of the treatment, there was a reduction of the gingival overgrowth associated with significant decreases of epithelium and connective tissue, as well as an increase of bone densities and a decrease of osteoclasts.

Conclusion: Within the limits of this experimental study, it can be concluded that the deleterious periodontal effects of cyclosporin A administration may be time-related side-effects.

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Cyclosporin A is a cyclic endecapeptide that was initially isolated from the fungus *Tolypocladium inflatum gams* (1). The use of cyclosporin A as an immunosuppressant has revolutionized organ transplantation, which has become the management of choice for many patients with chronic and life-threatening conditions (2). cyclosporin A has also been used for the treatment of type 2 diabetes, rheumatoid arthritis, psoriasis, multiple sclerosis, malaria, sarcoidosis and several other diseases with an immunological basis (3). The main side-effects of cyclosporin A include nephrotoxicity, hepatic dysfunction, neurological disturbances, hypertension and gingival overgrowth (1, 4). A common and serious side-effect of allogenic organ transplantation is osteoporosis, and cyclosporin A may contribute to its pathogenesis. There are convincing evidences showing that cyclosporin A causes increased bone turnover, with higher resorption than formation, resulting in bone loss (5, 6). cyclosporin A-induced osteopenia has been reported to be associated with an increased incidence of bone fractures (7).

Alterations of tissue metabolism caused by cyclosporin A may be dependent on many variables such as

patient gender, cyclosporin A dosage and serum level, and concurrent drug therapy (8–11). Another factor that must be considered is duration of therapy (12). A longitudinal study by Montebugnoli *et al.* (12) demonstrated the relevant role of time in reducing gingival overgrowth in heart-transplanted patients undergoing cyclosporin A therapy. These authors suggested that the gingival overgrowth necessarily developed in responders within 6 months from transplantation and in most subjects may be a time-related side-effect probably due to a progressive reduction in the sensitivity of the periodontium to cyclosporin A. Nevertheless, at this time we could find no other longitudinal study in the literature confirming this relationship. There is also a lack of studies exploring the effects of long-term cyclosporin A therapy on alveolar bone homeostasis.

Therefore, the objectives of the present study were to evaluate the effects of long-term therapy with cyclosporin A on the gingival tissue overgrowth and on the alveolar bone metabolism.

Materials and methods

Animals

Eighty male Wistar rats (*Rattus norvergicus albinus*) weighing 50 g were housed under similar conditions in cages with access to food and water *ad libitum*. The animals were randomly distributed into eight groups of 10 animals each. Four groups were treated with cyclosporin A (Sandimmun®, Novartis Pharma AG, Basileia, Switzerland) injected subcutaneously in a daily dose of 10 mg/kg body weight during the periods of 60, 120, 180 and 240 days. According to Wassef *et al.* (13) this dosage provides plasma peak and trough levels of cyclosporin A of 1000 and 750 ng/ml, respectively. Control rats from the other four groups were daily injected subcutaneously with saline (NaCl 0.9%). All rats were weighed weekly.

Blood collection and analysis

At the time of death, the rats were anesthetized with 0.1 mg/100 g body weight ketamine (Francotar®, Virbac do Brazil Ind. e Com. LTDA, São Paulo, São Paulo, Brazil) and 4–5 ml of blood was obtained by direct cardiac puncture in heparinized capillary tubes for immediate calcium measurements, using an ICA-1 ionized calcium analyzer (Radiometer Company, Copenhagen, Denmark); other blood samples were centrifuged and the serum stored at −70°C until assayed. Total serum alkaline phosphatase activity was measured colorimetricaly (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 experimental sample were calculated from this standard of Bayer units.

Histology techniques

After blood collection the rats were killed by an overdose of anesthesia and the mandibles were carefully removed and soaked in 10% formalin. 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 made on the buccal-lingual aspects of the whole 1st left and right molar and stained with hematoxylin and eosin. Each first lower molar has a mesial-distal diameter of approximately 1 mm, producing approximately 160 sections of 5 μm each.

Histometry

Linear measurements were made with the help of a Zeiss microscope at a magnification of 125 × using a Sigma computer program (Mocha, Jandel Scientific, San Rafael, CA, USA). From each tooth, 10 measurements were made in sections of 60-μm intervals each. For statistical analysis the mean from each animal was used, calculated from the 20 measures obtained from first right and left molars. Oral epithelium thickness and height and width of the connective tissue were measured according to Fischer and Klinge *et al.* (14), as shown in Fig. 1.

Bone volume

Volume densities of bone (V_b) and osteoclasts (V_o) were also estimated according to the method described by Shen *et al.* (15) The counting was performed with the aid of a Zeiss microscope at a magnification of ×200. A square lattice of 25 test points was projected into the ocular microscope with the use of a microvid system that connected the microscope to a computer. For each animal a total of 1200 points were counted distributed equally on apical, buccal and lingual regions (see Fig. 2). A square lattice has 25 points, and 16 sections were necessary to complete the stereometric analysis. The choice for analyzing a total of 1200 points

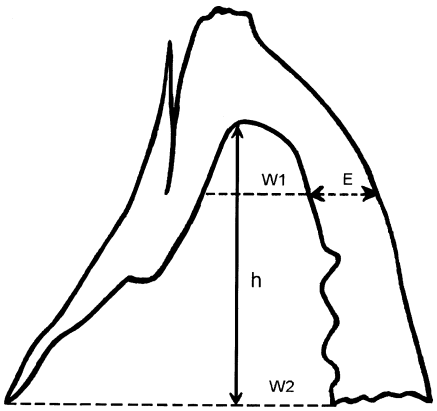

Fig. 1. Schematic illustration showing the regions where linear measurements were made. *E*, epithelium thickness; *h*, gingival connective tissue height; W_1 and W_2, connective tissue width.

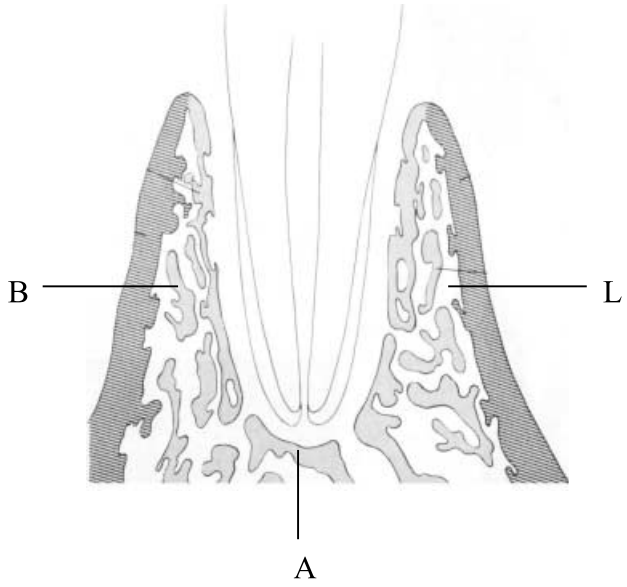

Fig. 2. Schematic illustration showing the regions where volume densities of bone and osteoclasts were measured. B, buccal region; L, lingual region; A, apical region.

was based on the relative error, described by Weibel (16), established in a pilot experiment of this present study. The distance between the selected sections was 60 μm. V_b and V_o were expressed as percentages of the total points counted.

Statistical analysis

Measurements were expressed as mean and standard deviation. Statistical analyses were made by the Tuckey test and Student's *t*-test.

Results

Serum data

Table 1 show the serum calcium and alkaline phosphatase levels of the control and cyclosporin A-treated rats. In the control group, the serum calcium levels ranged between 9.6 ± 0.3 and 9.9 ± 0.1 mg/dl. The cyclosporin A-treated groups showed a discreet decrease in the calcium levels; however, these values were not statistically different from those of the control group ($p > 0.05$).

A significant increase in serum alkaline phosphatase levels was observed in the groups treated with cyclosporin A compared to control groups. In the control group the serum alkaline phosphatase levels ranged between 824 ± 77 and 844 ± 71 U/dl and in the cyclosporin A-treated groups the levels ranged between 895 ± 75 and 908 ± 69 U/dl.

Histometric findings

Table 2 shows the linear measurements (μm ± SD) of the buccal gingival of the first lower molars of normal and treated rats. The gingiva of the control rats showed normal morphology and similar linear measurements at all periods of observation. The linear values of the cyclosporin A-treated rats were significantly increased at 60 and 120 days of treatment compared with the control group. At 180 and 240 days, the linear values in the cyclosporin A-treated rats decreased significantly and were not statistically different from those of the control group.

Stereometric findings of alveolar bone

Table 3 shows the volumetric densities of alveolar bone (V_b) from the buccal, apical and lingual region of the first mandibular molar in control and cyclosporin A rats. In control rats, the mean volumetric densities of bone were not statistically different from each other in the different periods of obser-

Table 1. Effect of cyclosporin A on serum calcium and alkaline phosphatase of the rats treated with cyclosporin A in different experimental periods

		Periods			
	Treatment	60 days	120 days	180 days	240 days
Calcium	Control	9.8 ± 0.1	9.6 ± 0.3	9.9 ± 0.2	9.9 ± 0.1
(mg/dl)	CsA	8.7 ± 0.2	8.9 ± 0.2	8.7 ± 0.2	9.2 ± 0.2
Alkaline phosphatase	Control	824 ± 77	840 ± 47	835 ± 63	844 ± 71
(U/dl)	CsA	895 ± 75*	888 ± 77*	901 ± 64*	908 ± 69*

Values present means ± SEM.
*$p < 0.05$, statistical significance vs. control rats in the same period. CsA, cyclosporin A.

Table 2. Linear measurements (μm ± SD) of epithelium thickness (E) and connective tissue height (h) and width (W) of the buccal gingival of the first right and left molars of normal rats and treated with cyclosporin A in various periods of treatment

		Periods			
	Treatment	60 days	120 days	180 days	240 days
E	Control	46.9 ± 0.4	44.5 ± 0.7	45.8 ± 1.4	47.9 ± 0.6
	CsA	102.1 ± 0.6[a]*	99.8 ± 0.7[a]*	56.3 ± 1.2[b]	55.4 ± 0.8[b]
h	Control	340.0 ± 1.2	341.2 ± 1.5	339.4 ± 1.4	336.7 ± 2.0
	CsA	692.4 ± 1.4[e]*	680.8 ± 1.6[e]*	440.0 ± 1.6[f]	387.6 ± 1.5[f]
W	Control	141.7 ± 1.0	146.9 ± 1.4	161.5 ± 1.9	143.4 ± 1.0
	CsA	313.4 ± 1.4[g]*	309.1 ± 2.3[g]*	172.3 ± 1.4[h]	154.1 ± 1.5[h]

W is the mean of W_1 and W_2 as shown in Fig. 1.
Different letters represent statistically significant differences among means in the same group.
*$p < 0.05$, statistical significance vs. control rats in the same period. CsA, cyclosporin A.

Table 3. Volumetric densities of alveolar bone (V_b) from the buccal, apical and lingual region of the first mandibular molar in control and cyclosporin A rats

		Periods			
	Treatment	60 days	120 days	180 days	240 days
Buccal	Control	84 ± 2	81 ± 3	89 ± 3	83 ± 4
	CsA	58 ± 4[a]*	54 ± 2[a]*	74 ± 3[a]*	80 ± 2[b]
Apical	Control	66 ± 8	68 ± 6	69 ± 4	70 ± 4
	CsA	45 ± 5[c]*	40 ± 6[c]*	53 ± 4[c]*	67 ± 4[d]
Lingual	Control	83 ± 3	84 ± 2	81 ± 4	84 ± 3
	CsA	53 ± 6[e]*	55 ± 8[e]*	61 ± 4[e]*	69 ± 4[f]*

Values present means ± SEM. The results are expressed as percentages.
Different letters represent statistically significant differences among means in the same group.
*$p < 0.05$, statistical significance vs. control rats in the same period. CsA, cyclosporin A.

Table 4. Volumetric densities of osteoclasts (V_o) from the buccal, apical and lingual regions of the first mandibular molar in control and cyclosporin A rats

		Periods			
	Treatment	60 days	120 days	180 days	240 days
Buccal	Control	2.5 ± 2.3	2.6 ± 1.8	4.3 ± 2.1	4.9 ± 3.7
	CsA	14.4 ± 2.8[a]*	14.8 ± 1.5[a]*	5.1 ± 3.7[b]*	5.9 ± 4.8[b]*
Apical	Control	3.3 ± 4.0	2.8 ± 4.2	5.7 ± 4.8	5.9 ± 5.1
	CsA	16.5 ± 4.3[c]*	14.9 ± 3.9[c]*	8.4 ± 5.2[d]*	6.3 ± 5.8[d]*
Lingual	Control	3.1 ± 2.2	4.2 ± 2.6	4.4 ± 3.9	4.0 ± 3.5
	CsA	11.9 ± 2.5[e]*	9.3 ± 3.3[e]*	6.2 ± 5.6[f]*	4.5 ± 4.1[f]*

Values present means ± SEM. The results are expressed as percentages.
Different letters represent statistically significant differences among means in the same group.
*$p < 0.05$, statistical significance vs. control rats in the same period. CsA, cyclosporin A.

vation. There was a significant decrease of bone volume at 60, 120 and 180 days of treatment in comparison to the control group. However, at 240 days, the percentages of bone volume in the cyclosporin A-treated rats increased significantly in the buccal and apical region and were not statistically different from those of the control group.

The volumetric densities of osteoclasts (V_o) from the buccal, apical and lingual region of the first mandibular molar in control and cyclosporin A rats are demonstrated in Table 4. There was a significant increase in the volumetric densities of osteoclasts in the cyclosporin A-treated rats when compared with those of the control rats. At 180 and 240 days, the volumetric densities of osteoclasts decreased significantly when compared with the initial experimental periods (60 and 120 days).

Discussion

The present study evaluated the gingival overgrowth and the alveolar bone alterations following long-term administration of cyclosporin A in a well-characterized animal model. The relevance of this study is the long period of observation of the effects of cyclosporin A on periodontium, as compared to other experimental studies (15, 17, 18) with briefer periods of time.

In agreement with previous studies (15, 17), this work showed that cyclosporin A administration, for 60 and 120 days, in doses that have been reported to be immunosuppressive in the rat, resulted in evident gingival overgrowth. The gingival overgrowth showed an increase of connective tissue and epithelial tissue compared with the respective control. Although the exact mechanisms involved in the development of the cyclosporin A-induced overgrowth are not known, there is substantial evidence that this drug acts directly or indirectly on the growth and function of both gingival fibroblasts and collagen fibers via cytokines and growth factors (19, 20).

Interestingly, in the present study, a gradual time-related improvement was observed regarding the modifications in the gingival volume at the longer periods of treatment (180 and 240 days). These results are in agreement with a recent prospective longitudinal study (12) that showed the relevant role of time in reducing gingival overgrowth in heart-transplanted patients undergoing cyclosporin A therapy from 6 to 48 months after transplantation. These authors suggested that the reduction in gingival overgrowth could be the result of a positive effect of time in reducing the sensitivity of the gingiva to the hyperproductive effects of cyclosporin A and could not be dependent of the plaque-control program. A time-dependent pattern of gingival overgrowth in nifedipine-treated animals was also demonstrated by Fu *et al.* (21). In their study, increasing gingival dimensions were observed from the 3- to 9-weeks observation interval. However, at longer observation intervals (6 to 9 weeks), it was difficult to demonstrate a further increase in overgrowth.

In the present study we also evaluated whether the effect of cyclosporin A on bone and mineral metabolism is dependent on the duration of treatment. In agreement with Schlosberg *et al.* (22) and other previous studies (17, 23–27), this present investigation showed that cyclosporin A administration resulted in striking and unique histomorphometric and serum changes in rat bone mineral metabolism at the earlier experimental periods (60 and 120 days). The data presented in this study indicate that cyclosporin A administration for 60 and 120 days leads to an increase in the volumetric densities of osteoclasts (V_o) and to a decrease in the volumetric densities of alveolar bone (V_b). 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 (28). 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 (29). The mechanisms by which cyclosporin A induces such osteopenia remain unclear,

although some hypotheses have been presented. Buchinsky *et al.* (30) 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 (31, 32) thus influencing bone remodeling (33).

A time-related improvement occurred at the longer periods of treatment (180 and 240 days) not only in the gingival volume but also in the volumetric densities of alveolar bone, associated with a significant decrease in the volumetric densities of osteoclasts. The reason for this effect is unknown, although we have hypothesized that the whole periodontium, rather than just the gingiva, may be influenced by possible mechanisms which lead to a reduction in the sensitivity to cyclosporin A with time.

The biochemical assessment of biomarkers of bone turnover correlated well with the stereometric findings. Our present experiment showed a significant decrease in serum calcium level, which, accordingly to Mason (34) and Ryffel (35), 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 (24, 25). In accordance with a previous study (36), a significant increase in serum alkaline phosphatase level, a marker of osteoblast phenotype, was also observed in the present study in cyclosporin A-treated groups, suggesting a modest positive effect of cyclosporin A on bone formation.

Within the limits of this experimental study, it can be concluded that the negative effects of cyclosporin A administration on the periodontium, characterized by gingival overgrowth and alveolar bone loss, could be improved with time. Nevertheless, detailed studies are still needed to clarify the possible cellular and molecular mechanisms involved in the diminished effect of immunosuppressive drugs on the periodontium after long-term administration.

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