Effects of long-term cyclosporin therapy on gingiva of rats – analysis by stereological and biochemical estimation

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INTRODUCTION

Cyclosporin A (CsA) is a cyclic undecapeptide that was initially isolated from the fungus Tolypocladium inflatum gams. The use of CsA as an immunosuppressant has revolutionized organ transplantation, which has become the management of choice for many patients with chronic and life-threatening conditions. CsA has also been used for the treatment of type 2 diabetes, rheumatoid arthritis, psoriasis, multiple sclerosis, malaria, sarcoidosis, and several other diseases with an

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immunological basis. CsA’s main side effects include nephrotoxicity, hepatic dysfunction, neurological disturbances and hypertension. The most prominent side effect of CsA therapy in oral tissues is gingival overgrowth.

Alterations in fibroblasts, together with increased collagen production and/or degradation, are considered to be the cause of this overgrowth. Alterations in tissue metabolism caused by CsA may be dependent on many variables such as patient’s gender, CsA dosage and serum level, and concurrent drug therapy.

Another factor that must be considered is the duration of therapy. A longitudinal study by Montebugnoli et al. (2000) suggested that gingival overgrowth occurs during periodontal disease and regression of CsA-induced gingival overgrowth during treatment time is not well understood at the moment. They are probably a consequence of disturbances in the homeostatic equilibrium between synthesis and degradation of extracellular matrix molecules or interference in the fibroblast proliferation rates. CsA not only blocks the immune system by means of inhibition signaling through the cell receptor, but also stimulates the signaling cascade.

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**MATERIAL AND METHODS**

Eighty (80) male Wistar rats (Rattus norvegicus albinus) which weighed approximately 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 ten animals each. Four groups were treated with CsA (Novartis Pharma, Basel, Switzerland), injected subcutaneously in a daily dose of 10 mg/kg of body weight during the periods of 60, 120, 180, and 240 days. According to Wassef et al. (1985), this dosage provides plasma peak and trough levels of CsA of 1,000 and 750 ng/ml, respectively. Control rats from the other 4 groups were daily injected subcutaneously with saline (0.9% NaCl) (Fresenius Kabi Ltda., Campinas, Brazil). All rats were weighed weekly.

**Salivation**

The animals were anesthetized with Francotar® (Virbac do Brasil Com. Ltda., São Paulo, Brazil) (1.25 g/kg of body weight IP). Salivation was stimulated with pilocarpine (1 mg/kg of body weight) injected intra-peritoneally (IP). Ten minutes after pilocarpine (Hexis Científica, Araraquara, Brazil) or saline injection, saliva was collected with a sterile syringe (Injex, Ourinhos, Brazil) (0.5 ml approximately). All samples were collected between 9 and 11 a.m. and stored at −20°C until assayed for the determination of TGF-β.

**Enzyme-linked immunosorbent assay (ELISA)**

The levels of TGF-β were measured by ELISA using the kits according to the manufacturer’s instructions (Endogen Company, New York, USA). Briefly, diluted saliva samples were added in duplicate to well plates coated with antibody and incubated at 37°C for 2 hours. After incubation, each well was aspirated and washed five times with wash buffer. After washing, peroxidase-labeled secondary antibody was added to each well and the plate was incubated at 37°C for 1 hour. After each wash was washed in a similar manner, the plate was incubated with tetramethylbenzene at room temperature for 20 minutes. The reaction was stopped by adding 1 N sulfuric acid. Optical density was measured at 450 nm using a spectrophotometer (Technicon, Domont, France). Sample concentration was assessed by means of a standard curve.

**Histology techniques**

The rats were killed by means of an overdose of anesthesia, and their mandibles were carefully removed and soaked in 10% formalin (Hexis Científica, Araraquara, Brazil). Decalcification was
carried out in 4.13% EDTA solution (Hexis Científica, Araraquara, Brazil) (pH 7.2) at 4°C for about three months. Five micrometer (5 µm) serial paraffin (Hexis Científica, Araraquara, Brazil) sections were made on the bucco-lingual aspects of the left and right 1st molars and stained with hematoxylin and eosin (Hexis Científica, Araraquara, Brazil). Each lower 1st molar had a mesial-distal diameter of about 1 mm, producing about 160 sections of 5 µm each. Histometric and stereological studies were made on the buccal gingiva.

**Histometry**

Gingival epithelium and connective tissue area measurements were made with the help of a Zeiss microscope (Carl Zeiss, Munich, Germany) at magnification of 125 X using a Sigma computer program (Mocha, Jandel Scientific, CA, San Rafael, USA). From each tooth, 10 measurements in sections of 60 µm intervals each were made. For statistical analysis, the mean value obtained for each animal was used, calculated using the 20 measurements obtained from the right and left 1st molars (Figure 1).

**Stereology**

Volume densities of fibroblasts (Vf), collagen fibers (Vcf) and other structures (Vo), i.e. blood vessels, nerves and unidentified structures, were estimated according to the principles established by Dellesse (1848), which were applied to histology by Weibel (1974). The count was performed with the help of a Zeiss microscope, using oil immersion at a magnification of 1,000 X. A square lattice of 25 test points was projected into the microscope ocular, with the use of microvid system (Cambridge Instruments Buffalo, New York, USA), which connected the microscope to a computer. For each animal, 16 sections were selected (eight from the left molar and eight from the right one), and 25 points were counted in each section. Vf, Vcf and Vo were expressed as percentages of the total points counted.

**Statistical analysis**

Data were expressed as means and standard deviation. ANOVA was used for statistical evaluation. Tukey’s test was used to compare differences between groups.

**RESULTS**

After 60 and 120 days of treatment with CsA, gingival overgrowth in all animals was observed. Gingival overgrowth was seen in all gingival areas, but it was more evident on the buccal gingival tissue of the lower molar teeth. In all CsA treated rats, the gingival epithelium was hyperplastic, with deep papilla interdigitations. The connective tissue was dense, and showed thick collagen fibers that were interspersed with delicate vessels and fibroblasts. After 180 days of treatment, gingival overgrowth was not observed.

**Level of TGF-β₁ in saliva**

The TGF-β₁ levels (ng/ml) of the control groups and of the groups treated with CsA are demonstrated in Table 1. The TGF-β₁ level in saliva was significantly higher (p < 0.05) at 60 and 120 days

![FIGURE 1 - Schematic drawing of area measurements (µm²) of epithelial tissue (E) and connective tissue (C). (T: tooth).](image-url)

| TABLE 1 - Level (± SD) of TGF-β₁ (ng/ml) in saliva in control groups and groups treated with CsA (Cyclosporin A). |
| --- | --- | --- | --- | --- |
| Treatment | 60 days | 120 days | 180 days | 240 days |
| Control | 24.56 ± 4.39 | 23.78 ± 4.44 | 23.02 ± 5.11 | 25.09 ± 4.11 |
| CsA | 49.98 ± 3.26 | 53.36 ± 4.19 | 30.04 ± 5.56 | 31.81 ± 5.41 |

SD: standard deviations.
of treatment with CsA when compared with control groups (mean values 49.98 ± 3.26, 53.36 ± 4.19 and 24.56 ± 4.39, 23.78 ± 4.44 respectively). After 180 and 240 days of treatment with CsA, the TGF-β level decreased (mean values 30.04 ± 5.56, 31.81 ± 5.41 and 23.02 ± 5.11, 25.09 ± 4.11), but levels were not similar to the levels observed in control groups (p < 0.05).

**Histometric findings**

Table 2 shows the area (µm² ± SD) of the epithelium and connective tissue of the buccal gingiva of the lower first molars of control and treated rats. The gingiva of control rats showed normal morphology and similar area at all periods of observation. The area values for epithelium and connective tissue were statistically higher (p < 0.05) at 60 and 120 days of treatment with CsA, when compared with control groups. After 180 and 240 days of treatment, the area values of epithelium and connective tissue were still higher when compared with control groups; however, with no statistically significant difference (p > 0.05).

**Stereometric findings**

Stereometric findings of the control groups and of the groups treated with CsA are demonstrated in Table 3. In the control groups, volumetric densities of fibroblasts, collagen fibers and other structures were, respectively, 11.87%, 66.66% and 21.47% in the buccal gingiva, and these values remained constant in all the studied periods. The stereometric alterations detected in the treated groups were similar in both gingiva. The volume densities of fibroblasts and collagen fibers increased after 60 and 120 days of treatment with CsA, while the volumetric densities of other structures decreased (p < 0.05). After 180 and 240 days of treatment with CsA, the volumetric densities of fibroblasts and collagen fibers decreased and were similar to values of the control groups, while the volumetric densities of other structures increased.

**DISCUSSION**

The present work evaluated the stereological and biochemical changes in gingival tissue following long-term administration of CsA in a well...

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<tr>
<th>TABLE 2 - Area (µm² ± SD) of gingival buccal epithelium and connective tissue of the lower first molars of normal rats, which were treated with CsA (Cyclosporin A).</th>
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<tbody>
<tr>
<td><strong>Buccal epithelium</strong></td>
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<td>CsA</td>
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<td><strong>Connective tissue</strong></td>
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<td>CsA</td>
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a - Significantly different from corresponding control groups (p < 0.05). b - Significantly different from 60- and 120-day treatment groups (p < 0.05). SD: standard deviations.

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<th>TABLE 3 - Volumetric densities of fibroblasts (Vf), collagen fibers (Vc) and other structures (Vo) in the buccal gingiva region of the mandibular first molar in control and CsA (Cyclosporin A) rats. Values present Means ± SEM. The results are expressed as percentages.</th>
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<tr>
<td><strong>Vf</strong></td>
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a - Significantly different from corresponding control groups (p < 0.05). b - Significantly different from 60- and 120-day treatment groups (p < 0.05). SEM: standard error of the mean.
characterized rat model. The rat has been extensively used to study the effects of CsA in the gingiva, and the resulting overgrowth is similar to that in humans. In fact, the response in rats is more uniform than in humans. There is agreement in the literature about the "risk factors" of gingival overgrowth. In humans, various "risk factors" associated with both the development and expression of CsA-induced gingival changes have been identified and quantified. These risk factors include age, sex, drug variables, concomitant medications, periodontal variables and genetic factors. Elucidation of such factors may help the treatment of "risk patients".

On the other hand, as suggested by Kataoka et al. (2000), many variables are better controlled in rats, such as genetic predisposition, gender, age and dose of CsA. The routes of administration and dosages of CsA that were used in this work gave consistent responses, as described by our group and by other authors. All the rats that were used in this experiment responded positively and uniformly to CsA treatment after short periods of treatment (60 and 120 days). In agreement with previous studies, in our study, gingival overgrowth showed increase of connective tissue and epithelial tissue when compared with control groups. Although the exact mechanisms involved in the development of CsA-induced gingival overgrowth are not well 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. Increased TGF-β production has been demonstrated in CsA-induced gingival overgrowth as well as in other fibrotic side effects of CsA-treatment. TGF-β is a multifunctional peptide that regulates diverse biologic activities including cell growth, cell death or apoptosis, cell differentiation, and extracellular matrix synthesis. TGF-β is believed to be a key mediator of tissue fibrosis as a consequence of extracellular matrix accumulation in pathologic states such as hereditary gingival fibromatosis and progressive renal diseases including CsA-induced nephropathy.

In the present study, a significant increase in the volumetric densities of fibroblast and collagen fibers after short treatment periods (60 and 120 days) was verified. These results are in accordance with results reported in the related literature and some previous studies performed by our group. However, some authors have not observed increased fibroblast density. Fibroblast heterogeneity remains one of the key factors used to explain the variable response of gingival tissue to the various gingival overgrowth-induced drugs. A genetic predisposition could also influence the metabolism of CsA. All these factors have already been observed in gingival overgrowth established or during its development.

It is important to point out that the purpose of this study was to investigate the level of TGF-β in saliva and describe the densities of fibroblasts and collagen fibers in the gingival tissue of rats treated with CsA for long periods, as compared to other experimental studies with shorter periods of time. Interestingly, in the present work, a gradual time-related improvement was observed, regarding the modifications in gingival volume after longer periods of treatment (180 and 240 days), with decrease in volumetric densities of fibroblasts and collagen fibers. This fact is not well documented in humans and has not been well explored in experimental studies.

Similar results were found by a recent prospective longitudinal study in humans that showed the relevant role of time in reducing gingival overgrowth in heart transplant patients undergoing CsA therapy from 6 to 48 months after transplantation. Recently, Spolidorio et al. verified that, besides the regression of CsA-induced gingival overgrowth, alveolar bone loss also decreases with time of treatment. Montebugnoli et al. (2000) suggested that the reduction in gingival overgrowth could be the result of a positive effect of time in reducing the sensitivity of gingival tissue to the hyperproductive effects of CsA, and could be unrelated to a plaque control program.

A time-dependent pattern of gingival overgrowth in Nifedipine-treated animals was also demonstrated by Fu et al. (1998). In their study, increased gingival dimensions were observed after 3- to 9-weeks of observation interval. However, at longer observation intervals (6- to 9-weeks), it was difficult to demonstrate a further increase in overgrowth. The results of the present work suggest that special attention should be given to the clarification of the mechanisms of action of CsA on fibroblast and collagen fiber proliferation in vitro and in vivo, as well as on protein synthesis and collagenolytic activity, mainly with longer periods of treatment with CsA. To the best of our knowledge, no previous investigation has analyzed the direct effect of CsA on TGF-β levels in rats treated for long periods with CsA. In this case, we have determined that CsA upregulates the production of TGF-β after short periods of CsA therapy and downregulates it after long periods of therapy.
Therefore, the present study revealed that there was a correlation between the decrease in fibroblast and collagen fiber volume and the decrease in TGF-β1 level.

The study presented here revealed that there was a dynamic process in gingival homeostasis capable of altering or modulating fibroblast function and consequently extracellular matrix production after long CsA therapy. The changes in TGF-β1 levels verified in rats treated for a long period with CsA could modulate MMP1 and MMP2 production and consequently collagen synthesis as well as altered fibroblast proliferation. Elevated expression and production of TGF-β1, associated with reduced expression and activity levels of matrix metalloproteinases, in particular MMP-1 and MMP-2, the most important enzymes associated with extracellular degradation and remodeling 4.

In summary, the results of the present work show that after 60 or 120 days of CsA treatment there was a significant increase in Vf and Vcf as well as a significant increase in TGF-β1. After 180 or 240 days, reduction in gingival overgrowth associated with significant decreases in the level of TGF-β1, and also decreased Vf and Vcf, were observed. The data presented here suggests that after long-term therapy, the decrease in the level of TGF-β1, might contribute to an increase in the proteolytic activity of gingival fibroblasts, favoring normal extracellular matrix synthesis 6. With the application of molecular techniques to analyze other growth factors, we hope that these facts can be better understood, thereby contributing significantly to clarify the interaction of gingival cells with CsA or products of its metabolism. The studies exploring the pathogenesis of CsA-induced gingival overgrowth in rats do not explore the time factor of treatment with CsA on gingival overgrowth. With the analysis of the regression of CsA-induced gingival overgrowth, its etiopathogenesis can obtain new and outstanding pulse with the analysis of metabolism and cellular behavior. The mechanisms involved in the regulation, synthesis and destination of cellular products, as well as of the external agents that influence the biology of fibroblasts and the action of genetic information in the activities of these cells in the presence of CsA or of other drugs that induce gingival overgrowth can be better understood.

In a near future, the mechanisms involved in the regulation, synthesis and destiny of cellular products will hopefully be known in depth, as well as the external agents that can influence the biology of fibroblasts and the action of genetic information on the activities of these cells in the presence of CsA or other drugs that can lead to gingival overgrowth.

CONCLUSION

The data presented here suggest that after long-term therapy, a decrease in TGF-β1 levels occurs, which might contribute to an increase in the proteolytic activity of gingival fibroblasts, favoring normal extracellular matrix synthesis.

REFERENCES