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Cyclosporine A – Induced gingival overgrowth and proliferating cell nuclear antigen expression in experimental periodontitis

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ABSTRACT

The most important microscopic characteristic of Cyclosporine A-induced gingival overgrowth is fibroepithelial hyperplasia. Objective: The objective was to investigate the influence of previous exposure to Cyclosporine A over gingival epithelium in experimental periodontitis in rats. Methods: Twenty Wistar rats with 12 weeks-old were divided into four groups with 5 animals each: Control Group (CG); Cyclosporine Group (CsAG); Ligature group (LG) and Cyclosporine and Ligature Group (CsALG). Daily doses of CsA (10 mg/kg) were applied to CsAG and CsALG during 60 days since the beginning of the experiment and, a ligature was placed in LG and CsALG 30 days after the beginning of the experiment. After 60 days, animals were euthanized and gingival tissue was processed to histomorphometric analysis of epithelial thickness (mm^2), immunohistochemical expression of PCNA (%) and inflammatory response. Data were analyzed by Kruskal-Wallis and Mann Whitney at 0.05 significance level. Results: Considering epithelial thickness, CG was thinner than all groups, CsALG was the largest and CsAG and LG were similar between each other. Regarding the PCNA expression CG (16.46 ± 9.26) was similar to CsAG (34.47 ± 19.75) and, LG (59.02 ± 10.33) was similar to CsALG (40.59 ± 18.25). Significant difference ($p < 0.05$) occurred only in inflammation presence comparing CG/LG and CsAG/CsALG. A weak positive correlation between the number of PCNA+ and inflammatory cells ($p = 0.001$; $r = 0.611$) was observed. Conclusion: Based on these results it was concluded that the enlargement of gingival epithelium observed in experimental periodontitis can be increased by previous exposition to CsA and inflammatory conditions enhanced proliferative activity of the keratinocytes.

1. Introduction

Cyclosporine A (CsA) is a hydrophobic, cyclic endecapeptide used as an immunosuppressive agent to prevent rejection of transplanted organs and in treatment of several diseases as type 1 diabetes, rheumatoid arthritis, psoriasis, malaria, erosive lichen planus, multiple sclerosis and ulcerative colitis. The use of this medication is associated with some side effects as liver dysfunction, renal and neuro–toxicity and gingival overgrowth.[1] Gingival overgrowth is characterized by a dense collagenous stroma and epithelial hyperplasia.[2]

Some studies has been pointed the connective tissue enlargement as the main factor of the gingival overgrowth. Nevertheless, other studies have been relating the epithelial hyperplasia as a frequent feature in this disease.[3] The etiology of CsA-induced gingival overgrowth still remains incompletely explained[4] and many risk factors have been associated with its development and progression.[5] The accumulation of dental plaque[6] and inflammatory mediators associated with periodontal disease[7,8] have been suggested as local factors of gingival overgrowth. The studies are not conclusive about the requirement of accumulation of dental plaque for the initial lesion or if it is a consequence of gingival overgrowth.

Some studies with experimental animals treated with CsA have been proved the development of gingival overgrowth similar to that observed in human being, so this model have been used to investigate the histopathological features of the gingival enlargement. In an experimental study, the medication was administered simultaneously to the periodontal disease induction in a rat model. The CsA induced attenuation of periodontal bone loss[9] however the authors did not mentioned if the

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gingival enlargement is associated with the inflammation condition or with the drug induction. On the other hand, patients post solid organ transplantation, under medication with Cyclosporine, demonstrated worse periodontal condition compared to patients without Cyclosporine, since immunosuppression changed prevalence of selected potentially periodontal pathogenic bacteria.[6]

The histopathological studies of CsA-induced gingival overgrowth have been demonstrated the enlargement of epithelial and connective tissue. However, most of them, investigated the influence of fibroblasts[10,11] and inflammatory cells on this process[12]; the importance of epithelial hyperplasia on gingival overgrowth have been infrequently studied. Literature about influence of CsA on the keratinocyte proliferation and differentiation had presented inconclusive results; some studies presented increased epithelial differentiation[13] while other demonstrated *in vitro* that CsA inhibits cell proliferation and suppresses cytokine gene expression in keratinocytes.[14]

In this context, the objective of this study was to investigate the influence of previous exposure to Cyclosporine A over gingival epithelium in experimental periodontitis in rats.

2. Material and methods

2.1. Animals and experimental design

Forty adult male Wistar rats (Rattus novergicus, variation albinos), weighing approximately 270 g, kept in cages with five animals and treated with water and food *ad libitum* were used. The study was conducted in accordance with the standards approved by the Brazilian College of Animal Experiments (COBEA) and was submitted to the Animal Research Ethics Committee of UNITAU (EAEC registry/UNITAU nº 0019/06). The rats were initially randomly separated into two groups (n = 20): a Control group (CG); a Cyclosporine A group (CsAG) that received administration of daily doses of 10 mg/kg of CsA (Sandimmun, Novartis Pharma Co. Ltd, Basel, Switzerland) by gavage during all experimental period. Thirty days after de beginning of the drug treatment each group was divided once more and half of the animals received an insertion of a cotton ligature, as previous described,[15] around of their upper second molar to create four groups (n = 10): CG; Ligature group (LG); CsA and, Ligature and Cyclosporine A group (CsALG). The duration of the complete experiment was sixty days, while the duration of induction of periodontitis occurred during the last thirty days. During euthanasia, the cardiac perfusion occurred under general anesthesia after using intramuscular injection of a combination of 13 mg/kg body weight 2% xylazine (Rompum, Bayer, São Paulo, SP, Brazil) and 33 mg/kg body weight of ketamine (Francotar, Virbac, Roseira, SP, Brazil). Samples from maxillary bones were removed, storage in formaldehyde 10% for at least 24 h, included in paraffin and submitted to standard histological technique to HE stain and immunohistochemical technique.

2.2. Histomorphometric analysis

After maxillas has been decalcified in 10% EDTA for 30 days, 5 µm and 3 µm semi-serial paraffin sections were cut in bucco-lingual direction trough upper second molar used for hematoxylin & eosin (HE) and immunohistochemical staining. Gingival area and epithelial area (mm^2) was measured in lingual gingival papilla using a light microscope (Axiolab Standard 20, Carl Zeiss, Jena, Germany) at a magnification of ×200. Measurement was made using the program Image Tool for Windows, version 3.0 (Image Tool, San Antonio, Texas, USA). Inflammatory condition was measured by counting of mononuclear inflammatory cells that was classified considering their shape as round cells which had nucleus filling most part of cytoplasm and strongly stained by hematoxylin. This analysis was made using program Image J for Windows, version 2.0, in a magnification of ×200. The mean of 5 sections was used for data analysis.[16]

2.3. Immunohistochemical analysis

Immunohistochemical analysis was performed using a biotin-streptavidin-peroxidase kit (LSAB kit, DAKO, Carpinteira, CA). Sections were deparaffinized in xylene and then rehydrated by serial incubations in ethanol. Tissue sections were pretreated by microwave heating in citrate buffer, pH 7.4, for 15 min. The specimens were incubated with the primary monoclonal mouse anti-Proliferating Cell Nuclear Antigen (PCNA) antibody diluted 1:75 (Clone: PC10, DAKO, CA, USA) overnight at 4 °C in a humid chamber. Sequential incubation then was performed with biotinylated secondary antibody and peroxidase-labeled streptavidin (DAKO). Staining was revealed with diaminobenzidine (DAKO). Sections were counterstained with Mayer's hematoxylin and mounted in synthetic resin. Section stained only without incubation of primary monoclonal anti-PCNA antibody was used as negative control. Lymph node sections were used as positive control. In each section, 300 epithelial cells were counted in the basal layer of epithelium with ×400 magnification. Cells were considered PCNA positive when its nucleus was staining with brown color darker than the background regardless of staining intensity. The index of proliferative activity was based in the percentage of PCNA positive cells per total of number of nucleated epithelial cells counted.[17]

2.4. Statistical analysis

Initially, the normality of distribution of data was tested and experimental groups were compared using Kruskal-Wallis and Mann-Whitney non parametric tests. These analyses were performed using a statistical software package (BioStat, 5.0 version, USA).

3. Results

3.1. Histomorphometric results

Histological aspects of each group concerning epithelial thickness could be observed and have been increased in CsAG, LG and CsALG when compared with control group. The occurrence of inflammatory condition and periodontal breakdown is noted in ligature groups (LG and CsALG) by the epithelial migration in apical direction occupying the surface of root cement below the enamel-cement junction and alveolar bone loss. Epithelial tissue showed an overlying irregular, multilayered, parakeratinized epithelium with variable thickness, which contained some elongated ridges penetrating deep in connective tissue. It had been seen in connective tissue collagen fibers bundles irregularly arranged and high number of vessels and infiltrating mononuclear inflammatory cells (Fig. 1).

The histomorphometric measurement of gingival area and epithelial area is expressed in Table 1. Considering both analysis, significant difference was observed ($p < 0.05$) and, the higher value was found in CsALG and the lower in CG. No significant increase was noted between CsAG and LG.

The immunohistochemical analysis showed PCNA + stained cells in the basal layer of the epithelium in a heterogeneous distribution in all groups (Fig. 2). The counting of PCNA-proliferative index demonstrated that CG had significant lower values when compared with CsALG and had no difference among CsAG, LG and CsALG. The analysis of inflammatory cells verified that they were infiltrated in the connective

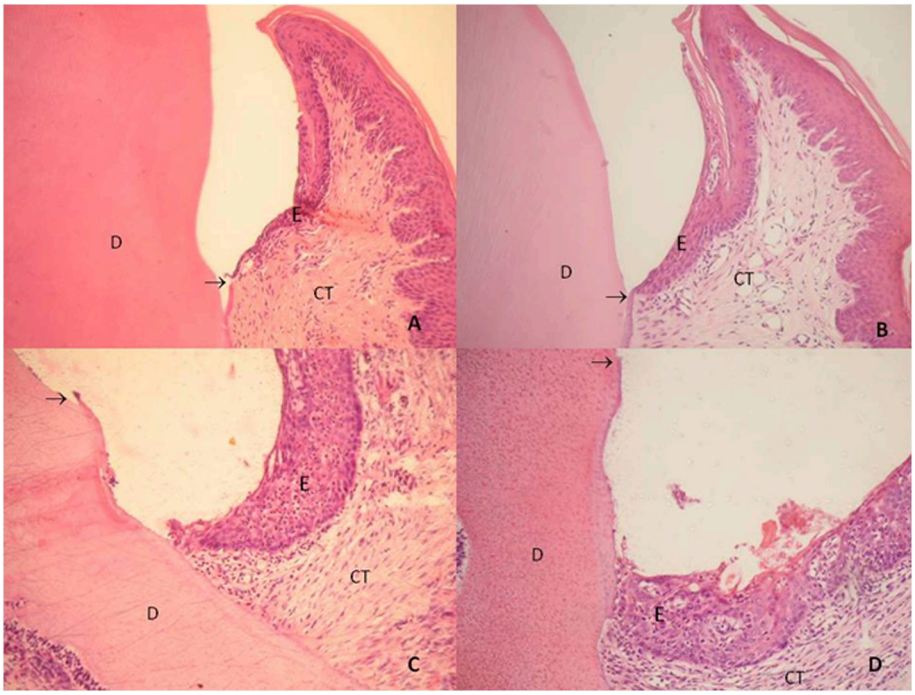

Fig. 1. Sections of each group showing the position of junctional epithelium (E) and the enamel-cement junction (→) and demonstrating de loss attachment in groups with ligature. A) Control group; B) Cyclosporine A treated group; C) Ligature group; D) Ligature and Cyclosporine A treated group. D = dentin; CT = connective tissue; E = junctional epithelium. H&E.

Table 1
Histomorphometrical measurements of gingival papilla and epithelial tissue of each group.

Groups	Gingival area	Epithelial area
CG	0.049 ± 0.018 a	0.038 ± 0.014 a
CsAG	0.097 ± 0.008 b	0.060 ± 0.011 b
LG	1.100 ± 0.033 b	0.053 ± 0.012 b
CsALG	0.145 ± 0.042 c	0.091 ± 0.017 c
P	0.0012	0.0001

CG; control group; CsA: cyclosporine A treated group; LG: ligature group; CsALG: cyclosporine A treated and ligature group. Same letters mean lacking of a significant difference.

tissue in all groups CG had the lower amount of inflammatory cell when compared to other groups (Table 2). The results showed a weak positive correlation between the number of PCNA+ and inflammatory cells ($p = 0.001$; $r = 0.611$) (Fig. 3).

4. Discussion

This study investigated the influence of previous exposition of CsA over the epithelial tissue in an experimental model of periodontitis in rats. This protocol using rats has been described in literature as an excellent model of studying gingival overgrowth as a side effect of the use of CsA.[18] The use of this animal model provided control of many variables associated with the host response as the inflammatory response and made possible to standardize the variables age, gender, time of exposition to the risk factor, and cyclosporine therapy.

The first topic analyzed was the gingival overgrowth measured by de area of gingival papilla. The results showed an enhanced area in groups with exposure to CsA and exposure to inflammation caused by experimental periodontitis. Association resulted in even more enhance area. These results were according with the literature.[18,19] Histological changes were expected in gingival overgrowth as an outcome of inflammatory host response to microbial dental plaque accumulation on the ligature in experimental periodontitis. These changes include vascular response which generates inflammatory cell infiltration and a plasmatic protein accumulation creating a tissue edema.

Some studies have associated CsA-induced gingival overgrowth with an increased activity of fibroblasts[20] which generates a hyperplasia of connective tissue. Our results are according with the literature that discuss that the gingival overgrowth can be related with inflammatory response. We demonstrated that the increase of epithelial thickness was strongly associated with enlargement of gingival papilla. This outcome is especially important when it is considered the possible effect of inflammatory mediators produced in specimens of LG and, how it could be modified in CsAL, once, in this group, the epithelium area in gingival papilla was larger than in CsA or LG specimens.

Some evidences have demonstrating that cyclosporine alters gingival epithelial cells, since proliferative activity of keratinocytes had been described decreasing[3,21] or increasing rates[22,23] according therapies and experimental models. One given explanation for diminishing keratinocyte proliferation is the low detection of DNA polymerase delta in immunohistochemical study of basal layer in CsA induced gingival overgrowth once it is essential for cell proliferation.[21] The histomorphometric results of Ayanoglou & Lesty proved that the increase of epithelial thickness was associated with a deficient keratinization of cells in CsA gingival overgrowth, suggesting that this enlargement of epithelial thickness was not only associated with epithelial cell proliferation, but also with its differentiation.[3] Conversely to this study, the results of Das et al. suggested that the exposition to CsA can modulate and amplify the expression of keratinocyte growth factor and stimulate differentiation and proliferation in gingival overgrowth.[22]

PCNA is a nuclear antigen that is associated with the proliferative state of cell. Cetinkaya et al.[24] observed that the immunolocalization of PCNA + cell was an efficient proliferating cell index and that, epithelial

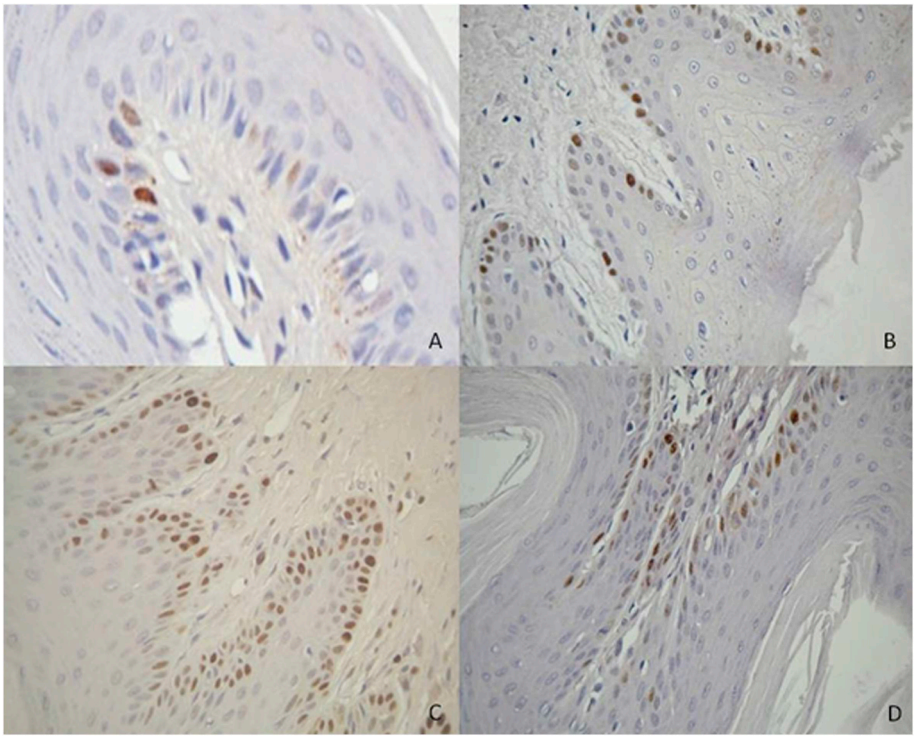

Fig. 2. Light microscopic views of immunostained sections showing the distribution of PCNA + cells in the buccal oral epithelium. × 400. (A) Control group CG; CsA-treated CsAG; Ligature group LG; CsA-treated and Ligature group CsALG.

Table 2
Immunohistochemical expression of PCNA+ and counting of inflammatory cells.

Groups	PCNA (+) cell	Inflammatory cells
CG	16.46 ± 9.26 a	110.62 ± 8.07 a
CsAG	34.47 ± 19.75 ab	203.00 ± 9.45 b
LG	59.02 ± 10.33 c	214.5 ± 37.23 b
CsALG	40.59 ± 18.25 bc	205.01 ± 25.75 b
P	0.003	0.001

CG; control group; CsA: cyclosporine A treated group; LG: ligature group; CsALG: cyclosporine A treated and ligature group. Same letters mean lacking of a significant difference.

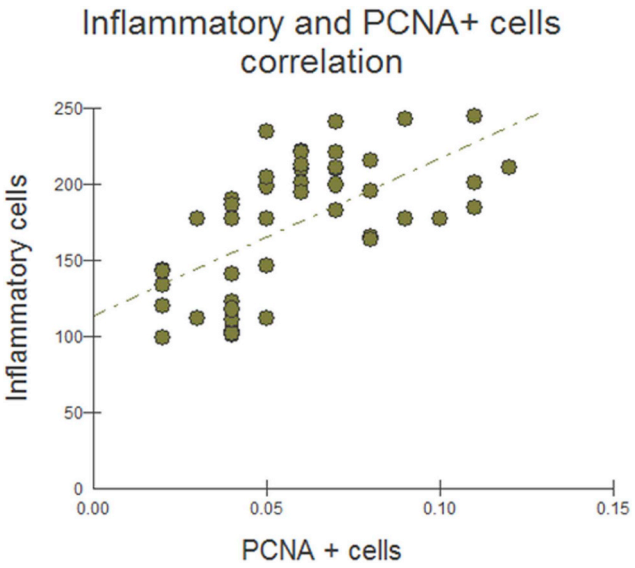

Fig. 3. Scatterplot graph of Pearson Correlation between the number of inflammatory cells and the PCNA + cells.

alterations in drug-induced gingival overgrowth was associated with increased proliferative activity. The results of the present study also showed increase of PCNA + cells in specimens of animals exposed to CsA but the significant change was observed in specimens that presented inflammatory response against dental plaque as in LG and CsAL, when compared with CG. This outcome can be supported by the results published in literature presenting the increase of epithelial thickness and of the expression of PCNA+ in biopsies of inflamed gingival tissue when compared with health gingival.[17]

At the same way, a study comparing the mitotic activity of keratinocyte between oral epithelium and sulcular epithelium demonstrated that the exposure to CsA was capable to influence the epithelial proliferation but the local inflammation had affected these drug-induced changes.[23] The association between inflammatory condition and drug-induced gingival overgrowth have already studied by Fu et al.[25]; authors detected that although enhanced gingival overgrowth occurred in a dose dependent way, this changes were more evident in ligature animals, suggesting that plaque retention magnifies CsA-induced gingival overgrowth. Authors also mentioned about the importance of dental plaque as a key cofactor in the development of CsA-induced gingival overgrowth.[25] The weak positive correlation between PCNA + cells and inflammatory cells observed at the present study corroborate with these literature results.

It was concluded that the enlargement of gingival epithelium observed in experimental periodontitis can be increased by previous exposition to CsA and inflammatory conditions enhanced proliferative activity of the keratinocytes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jobcr.2018.10.004.

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