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## Previous exposure to Cyclosporine A and periodontal breakdown in rats

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### ABSTRACT

**Objective:** The aim of this study was to evaluate the influence of previous exposure to Cyclosporine A (CsA) on experimental periodontitis in rats.

**Design:** Forty rats were divided into 4 groups: Control (CON); Cyclosporine A (CsA), which received daily doses of 10 mg/kg CsA; Ligature (LIG), which received an insertion of a cotton ligature around the mandibular 1st molar at day 30; and Ligature and CsA (CsAL), which were treated with CsA and the cotton ligature. At day 60 of the experiment, animals were sacrificed, and groups were compared with regards to Alkaline Phosphatase (AP) activity, gingival overgrowth, periodontal bone support (PBS), bone resorption at furcation ligament area (LA) and TRAP+ cells. Data were analyzed by ANOVA/Tukey and Kruskal–Wallis and were considered to be statistically significant at 5% level.

**Results:** CsA and LIG groups showed similar gingival area, which was higher than that in the CON and lower than in the CsAL group ( $p = 0.001$ ). The ratio between epithelial area and connective area for the CON group was similar to the CsA group and higher than that for the CsAL and LIG groups ( $p = 0.0334$ ). Mean percentage of PBS for the CON group was similar to that for the CsAL group and higher than that of the CsA and LIG groups ( $p = 0.0007$ ). No difference was observed regarding AP ( $p = 0.2806$ ) and TRAP+ cells ( $p = 0.3995$ ) among experimental groups. Mean values for LA of CON were similar to CsA, and both were statistically lower than the CsAL and LIG groups ( $p = 0.0172$ ).

**Conclusion:** Based on these results, we posit that previous exposure to CsA may influence gingival overgrowth, but not bone loss, in rats with experimental periodontitis.

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## 1. Introduction

Cyclosporine A (CsA) is currently the drug of choice to prevent organ rejection in patients undergoing organ transplantation. The drug is a potent immunosuppressant that is also indicated for the treatment of type 1 diabetes, rheumatoid arthritis, psoriasis, multiple sclerosis and ulcerative colitis.<sup>1</sup> The exact mechanism of action of the drug is still unknown, but it has been noted that CsA may interfere with the maturation of T cells and B cells by inhibiting production of interleukin 2 in the early stages of cellular activation.<sup>2</sup> The effectiveness of CsA in the treatment of some diseases resulted in its popularization and an increase in the number of prescriptions. However, some unwanted side effects have been associated with the use of the CsA, with those most commonly described in the literature being liver dysfunction, renal and neuro-toxicity, and gingival overgrowth. A number of changes in bone metabolism have been reported, which have been associated with osteopenia<sup>3–6</sup> and the pathogenesis of osteoporosis.<sup>7,8</sup> This finding, in association with the observed changes in epithelial and connective tissue, may represent a possible risk factor for periodontal disease. A number of hypotheses have been established to explain the pathogenesis of gingival overgrowth induced by CsA. However, the mechanism of induction is still unknown, and some questions remain unanswered.<sup>9,10</sup> Drug-induced gingival overgrowth, characterized by a dense collagen stroma and hyperplasia of epithelium, can occur in 13–80% of patients.<sup>11</sup> These changes in connective tissue were also associated with vasodilatation and increases in the volume of the extracellular matrix. However, enlargement of epithelial thickness, height and width of connective tissue, and increased density of fibroblasts and collagen have also been observed in some studies.<sup>9–11</sup> These studies were all designed applying CsA medication at the same time as the induction of periodontal disease. However, there is a lack of studies in which animals were immunosuppressed with cyclosporine first, followed by induction of periodontal disease to examine periodontal changes described in the literature. Therefore, the objective of this study was to evaluate the influence of previous exposure to CsA on experimental periodontitis induced in rats.

## 2. Materials and methods

### 2.1. Experimental design

Forty adult male Wistar rats (*Rattus norvegicus*, variation albinos) weighing approximately 270 g were housed with 5 animals per cage and provided with water and food ad libitum. The study was conducted in accordance with standards approved by the Brazilian College of Animal Experiments and was submitted to the Animal Research Ethics Committee (EAEC registry/UNITAU n° 0019/06). Rats were randomly separated into 4 groups ( $n = 10$ ): Control group (CON); Cyclosporine A group (CsA), which received administration of daily doses of 10 mg/kg of CsA (Sandimmun, Novartis, São Paulo, SP, Brazil) by gavage beginning at the start of the experiment for 60 days; Ligature group (LIG), which received insertion of a cotton

ligature around the mandibular 1st molar at day 30 after the beginning of experiment; and Ligature + Cyclosporine A group (CsAL), which received treatment with CsA and cotton ligature insertion. After 60 days, the animals were sacrificed by cardiac perfusion after intramuscular anaesthesia with 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).

### 2.2. Gingival overgrowth analysis

A fragment of gingival tissue from the lingual aspect of the mandibular 1st molar was collected from the mesial to distal gingival papilla. This surgical piece was inserted with the base in the bottom of the cassette for paraffin embedding, with sections done in the transverse direction in order to show connective and epithelial tissue.

Five semi-serial (5  $\mu$ m) sections per block were obtained and stained with haematoxylin and eosin, HE. Sections were photographed and scanned with the software Axiolab Standard 20 (Carl Zeiss Jena, Germany). The histomorphometric analysis of the total gingival area (TGA) was performed with the UTHSCSA Image Tool software version 2.0 (Image Tool, San Antonio, Texas, USA). The total area of fragment describing the gingival papilla first was calculated in mm. Then, the area of connective tissue was delimited. From these two measurements, the value of the area occupied by epithelium and the percentage of epithelium and connective tissue were calculated (Fig. 1).

### 2.3. Alkaline phosphatase analysis (AP)

Four to five ml of blood was obtained by direct cardiac puncture. Blood samples were then centrifuged at  $500 \times g$  for 10 min to obtain serum. AP catalyzes the transfer of a phosphate group in ambient alkaline from 4-nitrophenol to 2-amino-2-metil-1-propanol (AMP), yielding 4-nitrophenol. The extent of catalytic activity is determined by the speed of 4-nitrophenol measured at 405 nm. Kinetic methods used for the determination of AP activity followed Kit Labor LA recommendation (Gold Analisa Diagnóstica Ltda, Belo Horizonte, Brazil). AP concentration was expressed in U/L.

### 2.4. Radiographic analysis

The jaws were subjected to analysis using the digital radiographic imaging system Radiographic intra-oral Digital RVG<sup>®</sup> (RADIOVISIOGRAPHY–Trophy Radiology Inc. - Marietta/USA), which employs Charged Coupled Devices (sensor - CCD) to capture the images directly. The sensor was exposed to 65 kV and 7  $\mu$ A with a source-sensor distance of 30 cm. X-rays were taken with a digital x-ray apparatus 765DC Gendex<sup>®</sup> (Gendex, Dentsply, International, USA), with an exposure time of 0.08 s. Points were set for the measurement of periodontal bone support (PBS), according to Klausen, Evans and Sftintescu.<sup>12</sup> The first measurement was the distance between the portion of the more coronary cusp tooth to the tip of the ridge bone or bone defect, and the second measurement was the distance between the tip of the ridge bone or bone defect to the root apex. The PBS result was the ratio between



**Fig. 1 – Sections of gingival tissue showing measurement of epithelial area, connective area and total area. EO, External oral epithelium; C, Connective tissue; JE, Junctional epithelium. H&E stain in a magnification of 200×.**

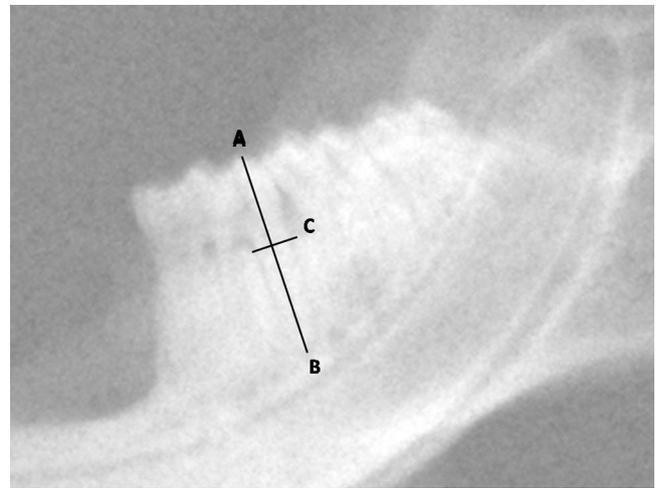
the first and second measure multiplied by one hundred. All measurements were performed with the software Image Tool for Windows version 2.0 (Image Tool, San Antonio, Texas, USA) (Fig. 2).

### 2.5. Bone resorption analysis

The mandibles were decalcified in 10% EDTA for 30 days. The samples were then embedded in paraffin with the lingual area faced to the cutting plane in order to obtain mesio-distal sections. Five semi-serial (5 μm) sections were prepared and stained with Mallory trichrome dye for the assessment of bone tissue at the furcation area. To analyze the bone resorption, periodontal ligament area (LA) was measured between the roof of the bifurcation of the mandibular left first molar and the crest of the bone septum inter-root using the software for playing images, Image Tool for Windows version 2.0 (Image Tool, San Antonio, Texas, USA) (Fig. 3).

### 2.6. TRAP analysis

The sections stained with the Acid Phosphatase Leukocyte Kit (Sigma–Aldrich, St Louis, Missouri, USA) were analyzed according to the marking-producing cells positive for tartrate

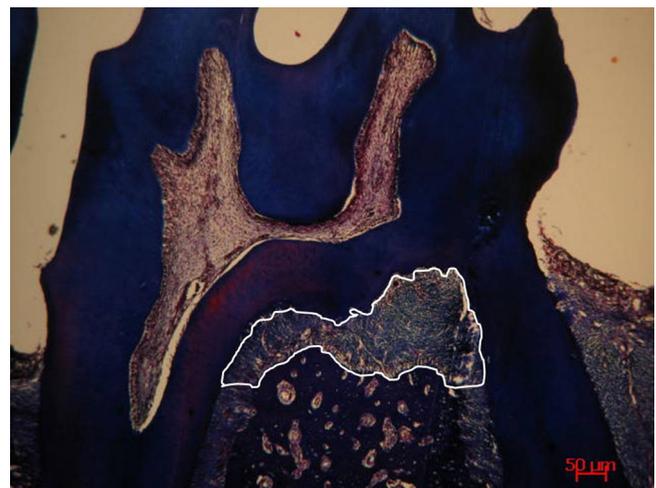


**Fig. 2 – Measurement of Periodontal Bone Support (PBS). (A) more coronary cusp tooth; (B) root apex; (C) tip of the ridge bone or bone defect.**

resistant acid phosphatase, stained in purple by a field observed in a light microscope. The sections considered to be weakly marked by TRAP+ cells received a score of one, the moderately marked cells received a score of two, and those strongly marked were given a score of three. This study was performed in duplicate by a calibrated examiner who was unaware of the content of the images.

### 2.7. Statistical analysis

Groups were compared according to the presence of CsA and the presence of inflammatory conditions. Data were statistically analyzed by a nonparametric test (Kruskal–Wallis) or a parametric test (ANOVA/Tukey) at the 5% significance level BioStat 5.0 software (Instituto de Desenvolvimento Sustentável Mamirauá, Tefe, Amazonas, Brazil).



**Fig. 3 – Sections of furcation region showing the periodontal ligament area. Masson Trichromic stain in a magnification of 100×.**

**Table 1 – Analysis associated with gingival overgrowth (mean  $\pm$  SD).**

	CON	CsA	LIG	CsAL
TGA <sup>#</sup>	0.05 $\pm$ 0.02a	0.08 $\pm$ 0.03b	0.10 $\pm$ 0.03c	0.14 $\pm$ 0.04c
EA/TGA <sup>#</sup>	71.39 $\pm$ 8.64a	67.27 $\pm$ 14.13ab	63.37 $\pm$ 7.44b	61.49 $\pm$ 10.98b
CA/TGA <sup>#</sup>	28.61 $\pm$ 8.64 a	32.73 $\pm$ 14.13ab	37.70 $\pm$ 7.49b	38.51 $\pm$ 10.98b
EA/CA <sup>#</sup>	2.80 $\pm$ 0.80a	2.18 $\pm$ 1.32ab	1.81 $\pm$ 0.80b	1.89 $\pm$ 1.17b

<sup>#</sup> Analyses by Kruskal–Wallis test ( $p < 0.05$ ); same letters means lacking of significative difference; TGA: total gingival area ( $\text{mm}^2$ ); EA/TGA; percentage of TGA occupied by epithelium (%); CA/TGA: percentage of GTA occupied by connective tissue (%); EA/CA: ratio of epithelium and connective tissue in TGA.

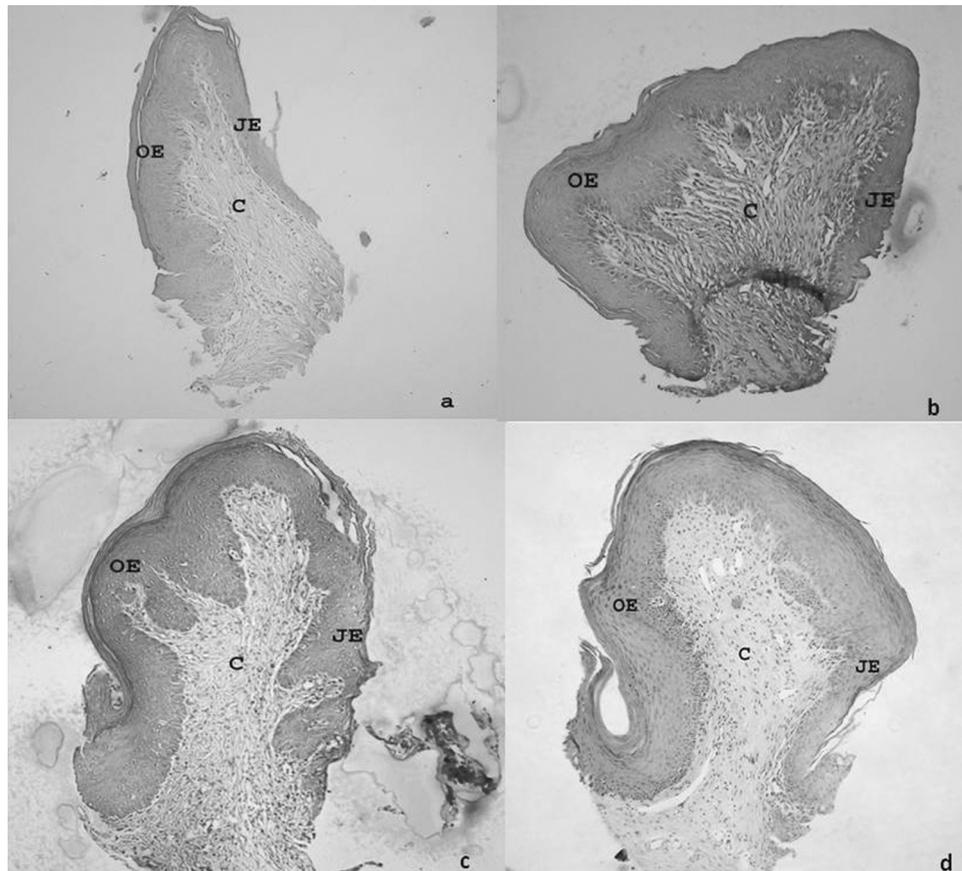
### 3. Results

#### 3.1. Gingival overgrowth

The results associated with gingival overgrowth showed differences among the experimental groups. Total gingival area for the CsA group was higher than for the CON group. There was no difference between the LIG and CsAL groups, and both were higher than the CON and CsA groups (Table 1 and Fig. 4). The tissue dimensions that were increased were connective tissue and total gingival area in CsA-treated groups (CsA and CsAL).

#### 3.2. Bone resorption

The results associated with bone resorption showed no difference among the groups for alkaline phosphatase ( $P > 0.05$ ). CsA and LIG groups showed significantly lower periodontal bone support compared to CON ( $P < 0.05$ ), and there was no difference between the CsAL group and any other group ( $P > 0.05$ ). Considering the average values of the ligament area, it was observed that the values for CON and CsA were different from those for LIG and CsAL ( $P < 0.05$ ), but with inflammation absent (CON and CsA) or present (LIG and CsAL) there were no differences ( $P > 0.05$ ) (Fig. 5). There were



**Fig. 4 – Sections of gingival tissue showing gingival enlargement (H&E stain in a magnification of 200 $\times$ ). Junctional epithelial (JE) and oral epithelial (OE) thickness and connective tissue (C) were increased in CsA compared to CON but no difference was observed when inflammatory conditions were associated with CsA. (a) Control group; (b) Cyclosporine A group; (c) Ligature group; (d) Ligature and Cyclosporine A group.**

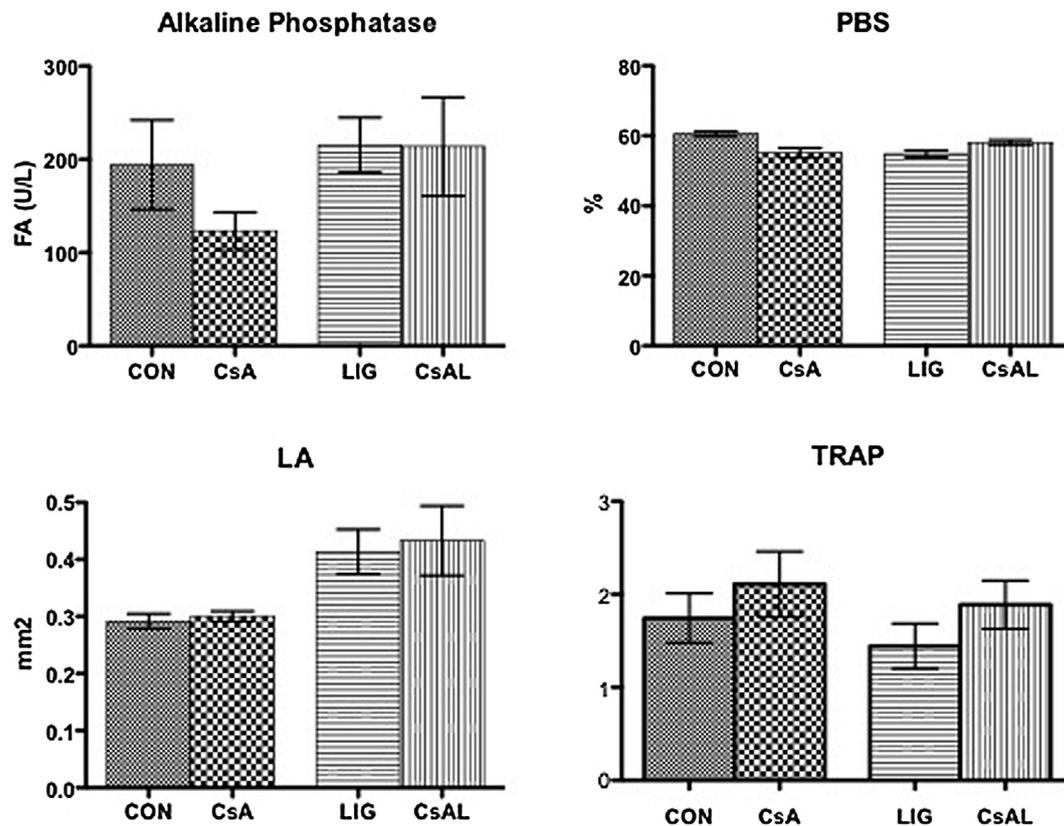


Fig. 5 – Analysis associated with bone resorption (mean  $\pm$  SD). AP, alkaline phosphatase (U/L); PBS, periodontal bone support (mm); LA, ligament area (mm<sup>2</sup>); TRAP+, tatarate resistant acid phosphatase positive cells (scores).

no statistically significant differences among experimental groups regarding the average values of TRAP+ cells (Fig. 6). Periodontal breakdown was characterized by enlargement of the LA and alveolar bone resorption. The same characteristics were observed in the LIG and CsAL groups.

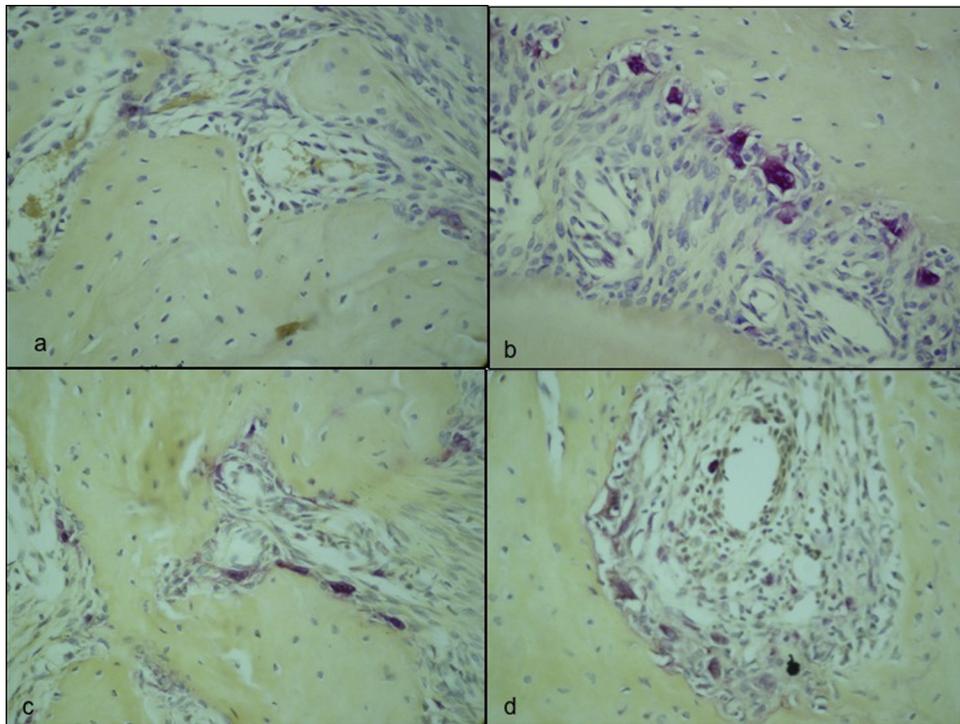
#### 4. Discussion

Induction of periodontal disease through the insertion of a ligature around the tooth in animals previously subjected to the use of CsA for a period of 30 days, as described in CsAL group, follows a different methodology from most of the studies described in literature in which the induction of the disease occurs simultaneously with the use of CsA.<sup>8,13,14</sup> The present protocol is based on the hypothesis that tissue alterations triggered by the induction of periodontal disease in animals could be modified by CsA mechanisms of action on tissue metabolism. In the present study, gingival total area (GTA) showed higher mean values in experimental groups, suggesting an association between inflammatory condition and cyclosporine (CsA, CsAL and LIG) as a potential factor for gingival overgrowth. The exact mechanism involved in this change is not yet fully understood.<sup>1,2</sup> Gingival enlargement induced by CsA may be a result of vasodilatation, which increases the volume of extracellular matrix as a result of increased epithelial thickness.<sup>9,15,16</sup> Nevertheless, according to the literature, the main feature of gingival overgrowth

induced by CsA is a decrease of collagen phagocytosis, which is responsible for greater proportion of connective tissue compared with total area of gingival tissue.<sup>9,17,18</sup> In addition, some reports were aimed at investigating the effect of CsA on the connective growth factor expression in human gingival fibroblasts and the potential chemopreventive agent for CsA-induced gingival overgrowth. Wu et al.<sup>19</sup> used western blot analysis to examine such pathways and showed that CsA significantly stimulated connective growth factor synthesis in human gingival fibroblasts.

The increase observed after association with inflammatory process (LIG and CsAL) may be related to characteristic biological events of periodontal disease pathogenesis of cell proliferation, blood vessels and oedema formation.<sup>20</sup> Epithelial tissue also showed increased thickness based on an increase of basal stem cell proliferation stimulated by CsA and inflammatory conditions.<sup>21</sup>

A study to investigate the effect of periodontal treatment in renal transplant recipients cited that the study results could support the hypothesis that plaque is a significant contributing factor in the development of cyclosporine-induced overgrowth.<sup>22</sup> Additionally, the results show that in patients without periodontal therapy, complications of cyclosporine had adverse effects on oral and periodontal health due to development of clinical overgrowth, in contrast to patients who had periodontal treatment. It is very interesting to observe the real importance of CSA induced overgrowth. Indeed, no gingival overgrowth was observed after periodontal



**Fig. 6 – Sections of gingival tissue showing the Trap staining. (a) Control group; (b) Cyclosporine A group; (c) Ligature group; (d) Ligature and Cyclosporine A group.**

treatment in patients who were administered CsA after renal transplantation, and good oral hygiene with good plaque control is the best prevention for gingival overgrowth.<sup>22,23,24</sup>

One mechanism of analyses of periodontal disease-related bone changes is the quantification of periodontal bone support, which can be used to investigate the influence of modifying risk factors on bone resorption in periodontal disease.<sup>8,18</sup> In this study, decreased radiographic PBS was noted in the LIG and CsA groups. It is possible to consider that reduction of PBS was directly related to induction of periodontal disease in animals.<sup>25,26</sup> The use of CsA alone may have promoted changes in bone tissue metabolism and, therefore, may have generated PBS reduction.<sup>3,4,26,27</sup> There is the possibility of bone resorption reduction in animals in which periodontal disease was induced at the same time of exposure to CsA could be a result of inhibition of T cells by CsA during the initial stages of periodontal disease induction in rats.<sup>8</sup> Jayasheela and Mehta<sup>28</sup> studied an experimental model in rats to evaluate the effects of CsA targeting the tissue of periodontal tissues. They divided the rats into test (CsA) and control (saline) groups. At the morphometric examination, a significant gingival overgrowth was observed in the CsA groups, while no evidence of this phenomenon was present in the saline group. Additionally, a significant loss of bone mass was observed in the test group compared with the control. Based on these results, they concluded that the CsA targets the periodontal tissues.

However, the results of the present study were similar to the study by Cetinkaya in 2006, which showed a decline in the process of bone resorption in rats with ligature-induced periodontal disease submitted to the simultaneous use of CsA, and no difference was found in radiographic and

histometric findings between the CsA and CsAL groups, or between the CsA and control groups.<sup>13</sup> Another tool for evaluating the biological events of periodontal disease is the measurement of periodontal ligament area at the furcation region in which periodontal disease was induced.<sup>14,29</sup> The present study showed that for the groups with induced periodontal disease (LIG and CsAL), LA was similar to one another and higher than in groups without inflammatory process (CsA and CON). Changes were more associated with the presence of inflammation than with the drug action over bone metabolism.

The process of bone remodelling is closely related to cellular activity of osteoclasts that participate in the process of bone turnover and play a key role in bone resorption.<sup>30</sup> To investigate bone remodelling mechanisms, TRAP+ cells and AP activity were analyzed. TRAP is a histochemical marker of monocytes and macrophage lineage cells related to bone resorption and is able to recognize the process of cell differentiation. Although a marked trend towards higher scores of TRAP+ cells was observed in the CsA, LIG and CsAL, the exposure to CsA did not generate any significant change in this process. Some studies have linked the adverse effects of osteoporosis and osteopenia to use of CsA.<sup>4,5,21,30,31</sup> These features regarding alveolar bone metabolism would be associated with increased osteoclastic activity and reduction of bone formation in the presence of CsA. In this study, the authors suggested that CsA immunosuppression might have caused a decrease in periodontal breakdown.<sup>30</sup>

These results can be explained by the influence of CsA on blockage in osteoclastic cells activity through inhibition of IL-1 production and, therefore, over bone formation process.<sup>8</sup>

However, some studies have showed an increase in the number of osteoclasts associated with the use of CsA and induction of periodontal disease in animal models.<sup>32,33</sup> The alkaline phosphatase enzyme is related to osteoblast activity in the physiological process of bone remodelling, and although lower values of enzyme activity had been observed in the groups subjected to the use of CsA (CsA and CsAL), no significant difference was observed among experimental groups. The literature shows contradictory results related to the influence of CsA exposure on AP activity. Although Nassar<sup>8</sup> observed no significant decrease in the level of this enzyme activity, suggesting that CsA would have a negative role in bone formation, Spolidório<sup>30</sup> observed an increase in the activity of this enzyme, suggesting the involvement of CsA in the bone formation process.<sup>30</sup>

Another important question is how to note the role of TGF- $\beta$ , a growth factor that has an important role in the pathogenesis of CsA-induced overgrowth. Indeed, it can be concluded that CsA stimulated connective growth factor expression production in human gingival fibroblasts, with the connective factor acting as a cofactor with TGF- $\beta$  to induce the maximal profibrotic effects.<sup>19</sup>

Based on the limits of the present study, it is possible to suggest that previous exposure to CsA may influence the gingival overgrowth, but not bone resorption in ligature-induced periodontitis in rats.

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## Competing interests

All authors declare no conflict of interest.

## Ethical approval

Not required.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.archoralbio.2015.01.004>.

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