Dental Research Journal

Original Article

The role of cyclosporine A on the periodontal tissues

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ABSTRACT

Background: Cyclosporin A (CsA) is a known immunosuppressive agent and can be considered as a lifesaving drug in the organ transplantation cases. However, it is associated with many side-effects on different tissues and body organs including the periodontal tissues. The present animal study was undertaken to evaluate the effects of CsA targeting the tissue triad of periodontal tissues, i.e., gingiva, alveolar bone and cementum in rats.

Materials and Methods: Twelve 6-week-old male Wistar rats weighing 150-200 g were considered for the case-control study in rats. The rats were divided into 2 groups: (1) CsA (test) group (2) Saline (control) group and were administered the same subcutaneously daily once for 45 days. Impressions were taken and study casts were prepared on weekly basis for the morphometric analysis. At the end of 45 days, rats were sacrificed and specimens were analyzed for histomorphometric analysis. CsA and saline groups were analyzed to test of association using the Student *t*-test at 99% confidence interval.

Results: The morphometric examination showed significant gingival overgrowth in the CsA group, whereas no such growth in the saline group. Similarly, on histomorphometric analysis, there was a significant loss of alveolar bone in CsA group as compared with control. Furthermore, there was large amount of cementum formation accompanied by insertion of new connective tissue fibers especially in the cervical region of the tooth in CsA group rats.

Conclusion: CsA targets the periodontal tissues (gingiva, alveolar bone and cementum) in different pattern. Its role in cementogenesis can be utilized for periodontal regeneration, if its local application is testified and verified in the future animal studies.

Key Words: Alveolar bone loss, cyclosporin A, gingival overgrowth, new cementum formation

Received: November 2012
Accepted: May 2013

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INTRODUCTION

Cyclosporin A (CsA) is one of the basic immunosuppressive drugs widely used to prevent organ transplant rejection and for the treatment of other immune diseases, such as Behçet's disease, rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease, ulcerative colitis and insulin dependent diabetes mellitus. The side-effects of CsA therapy include gingival overgrowth (GO), nephrotoxicity, hepatotoxicity and bone loss.[1] Periodontium is a target tissue for cyclosporine therapy wherein it targets specially the tissue of the periodontium (gingiva, alveolar bone and cementum) in a very unique way. Though CsA has a negative impact on gingiva (overgrowth) and alveolar bone (bone loss),[2] it stimulates cementum formation on the root surfaces.[3] The CsA-induced GO has been reported in both animal models[4,5] and human clinical cases.[6] The prevalence of CsA-induced GO, which affects the quality-of-life of patients, ranges from 8% to 70% with mean prevalence of 35%.[6]

Bone loss is a clinically important problem affecting 6% to 15% of patients in 1st year after organ transplantation. An increased alveolar bone destruction at periodontal sites was found in rats

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after CsA exposure.[7] This phenomenon of increased alveolar bone loss may be related to the current concept of "osteoimmunology" wherein there is an increased recognition of the interactions between cells of immune and skeletal systems.[8] There is accumulating evidence that CsA has a negative impact on bone metabolism in human,[9-11] although the current literature regarding the effect of CsA on alveolar bone is controversial and inadequate.

Dental cementum is a mineralized tissue covering the root dentin and plays a key role in the attachment of the tooth to the surrounding structures. This attachment occurs by means of functional insertion of periodontal collagen fibers into the new cementum (NC). In a recent animal study, it was observed that the cyclosporine fed rats showed NC deposition on the root surfaces with functionally inserting collagen fibers.[3,12] The NC is deposited on the afibrillar extrinsic fiber cementum, mainly on the cervical third of roots as irregular layers, spurs of various sizes and shapes or in both configurations.[3]

There are several studies in the literature investigating the role of CsA on the periodontal tissue triad on the individual basis.[4-8,12-14] However, on literature search and to the best of our knowledge, there is no such study, in which the impact of CsA on all the three periodontal target tissues were investigated simultaneously. Hence, the present case-control animal study was undertaken to investigate the effect of cyclosporine therapy to measure the GO morphometrically and evaluate the alveolar bone loss and NC formation histologically in Wistar rats.

MATERIALS AND METHODS

Selection of rats and care

Twelve male Wistar rats, age 6 weeks and weighing approximately 150-200 g were used for this case-control animal study. Rats were housed in polypropylene cages using husk as their bedding and kept in the animal house of the JJM Medical College, Davangere. The animal house temperature was maintained at 24°C and light cycle (12 h light and 12 h dark). Rats were fed with a standard commercial laboratory diet and water *ad libitum*. Handling of the rats was carried out as per the guidelines given by National Centre for Laboratory Animal Sciences. The ethical approval was obtained from the Ethical Committee of JJM Medical College, Davangere.

CsA administration

After 1 week of acclimatization in the animal house, the rats were randomly divided into two groups: six rats in each group. The control group rats were injected with normal saline 10 mg/kg body weight/day subcutaneously and test groups with 10 mg/kg body weight/day of CsA subcutaneously for a period of 45 days. CsA was procured from Novartis India and was injected using a tuberculin syringe. The rats were weighed once a week in order to adjust the dose of CsA. After the injections, the rats were given free access to food and water.

Assessment of GO

Impression making and preparation of stone models

To record the gingival changes in the mandibular anterior region, impressions were made prior to start of the study with customized acrylic trays and stone casts were poured to serve as baseline. The procedure was carried out at the end of 45 days and stone casts were poured. Gingival dimensions on the cast were measured.

Morphometric measurement of GO

The gingival dimensions in the region of mandibular incisors were measured and recorded [Figure 1]. Buccolingual dimension [BLi], mesiodistal dimension [MDi] and vertical height dimensions [VHi] in the

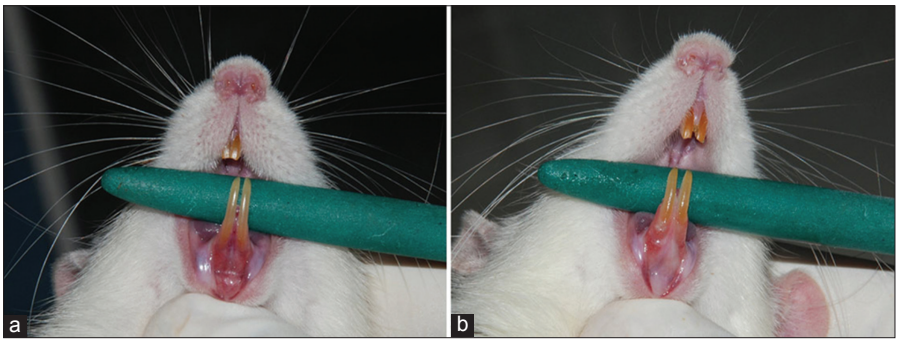

Figure 1: Gingival dimension in (a) saline treated rats (b) cyclosporin A treated rats

region of the interdental papilla was recorded as previously described.[14]

Assessment of alveolar bone loss by histomorphometric analysis

Rats were sacrificed at the end of the study period. The mandibles were carefully removed and the sections involving the mandibular molar teeth and bone were dissected out and immediately transferred into a solution containing 10% neutral formalin (a tissue fixative agent) and samples were subjected to histomorphometric analysis.

Morphometric analysis

To measure the bone loss morphometrically, images of the specimen were captured using a three chip charge coupled deviceCCD camera attached to a stereomicroscope with 5× objective. The measurement was carried out using the image pro-plus software. The distance was measured from the cusp tip to the crest of the alveolar bone.

Assessment of NC formation

Decalcification procedure

Decalcification of the specimen was carried out using the decalcifying agent (formalin-nitric acid solution) and this procedure was carried out in room temperature of 37°C. Each 100 ml solution consisted of formic acid-10 ml, Nitric acid-10 ml and distilled water-80 ml.

The solution was replaced every 3 days, whereas the completion of the decalcification was tested every day using "needle test." The test was carried out the using a sharp needle; when the needle penetrates the bone without any pressure it confirms the end of decalcification process (approximately 30 days). After the decalcification was carried out, the specimen was washed first in tap water overnight, then in the following order; 70% alcohol for 30 min, 80% alcohol for 1 h, 90% alcohol for 2 h, absolute alcohol for overnight and finally treated with chloroform for 2 h.

Tissues were embedded in paraffin wax and cut into slices in the thickness of 5 micron using a hard-tissue microtome. Tissue sections were subjected to H and E staining and viewed under stereomicroscope.

Statistical analysis

Statistical analysis was performed; CsA and saline groups were analyzed to test of association using Student *t*-test at 99% confidence interval.

RESULTS

In the present animal study, the morphometric analysis showed no GO in the saline treated rats [Figure 1a] where as significant ($P < 0.001$) increase in gingival dimensions in both maxillary and mandibular regions in the CsA treated rats [Figure 1b]. Furthermore in CsA treated rats, the increased size of the gingiva (both marginal and interdental papilla) produced diastema formation between mandibular anterior teeth. The change in the gingival dimension between CsA treated and saline treated rats was statistically significant with the P value of < 0.0001 [Table 1].

Morphometric evaluation of the dissected mandibular area revealed a significant amount of alveolar bone loss in the CsA group rats when compared to the saline treated rats [Figure 2]. However, some areas also showed concomitant alveolar bone formation and resorption. On histological examination, the alveolar bone resorption as measured from the cusp tip to the alveolar crest was found to be statistically significant ($P < 0.01$) [Figure 3] in the CsA treated rats as compared with saline treated rats. Such resorption sites were more pronounced and frequently seen in the CsA treated specimen than in the saline treated specimen [Figure 4].

The histological examination also revealed darkly stained thick layer of massive NC on the root surfaces in the CsA treated rats as compared to saline treated rats. Acellular type of cementum covered the coronal root dentin of the mandibular first molars of CsA group rats, whereas cellular cementum was more evident on the

Table 1: Change in the gingival dimension (in millimeters) between saline treated and CsA treated rats with standard deviation and *P* value from *t* test

Sl. no	Gingival dimension in saline treated rats (measured in millimeter)			Gingival dimension in CsA treated (measured in millimeter)		
	Bli	Mdi	Vhi	Bli	Mdi	Vhi
1						
2	2	1.3	0.7	3.1	2.4	3.2
3	2.1	1.7	1	3.2	2.3	2.3
4	2	1.6	0.9	2.9	2.2	2.4
5	1.9	1.6	1	3.1	2.4	2.4
6	2.1	1.7	1	2.7	2	2.5
Average	2.03	1.55	0.9	2.93	2.36	2.56
SD	0.082	0.16	0.13	0.24	0.30	0.33
t-test *P* value	0.000624	0.005369	0.000248			

CsA: Cyclosporin A

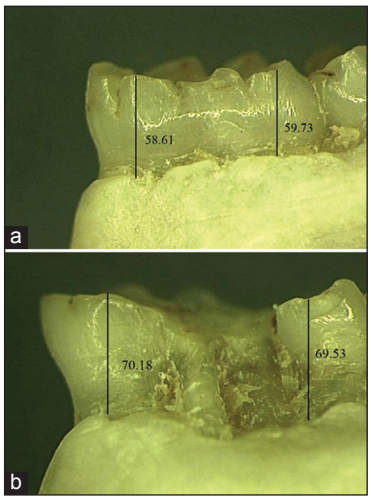

Figure 2: Morphometric assessment of alveolar bone level (a) no change in saline treated rats (b) reduced in cyclosporin A treated rats

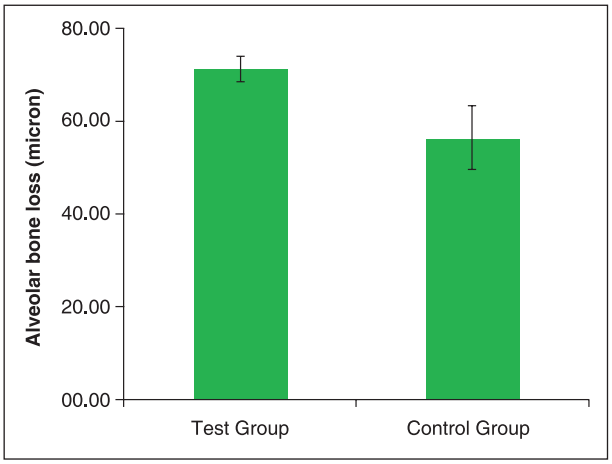

Figure 3: Graph showing alveolar bone loss in cyclosporin A treated and saline treated rats

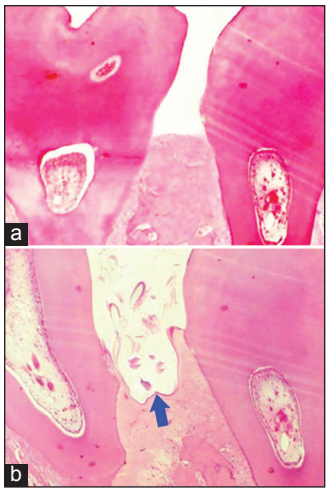

Figure 4: Histological assessment of alveolar bone level (a) no change in saline treated rats (b) reduced in cyclosporin A treated rats measured in microns

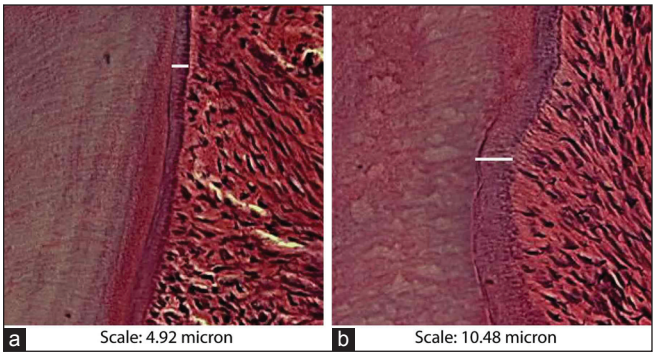

Figure 5: Thickness of new cementum (a) no change in saline treated rats (b) increased in cyclosporin A treated rats

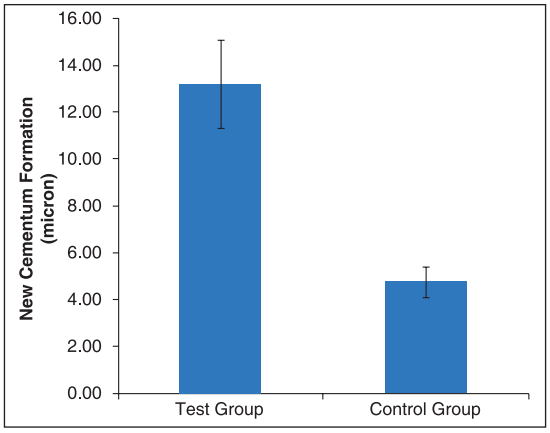

Figure 6: Graph showing new cementum formation in cyclosporin A treated and saline treated rats

apical third of roots. In some segments, the cementum consisted of two or more layers; the external layer showed a striated structure and presented suggestive images of inclusion of collagen fibers originating from the underlying connective tissue. The adjacent gingival connective tissue was composed of large amounts of dense collagen bundles inserting into the NC [Figure 5].

The cementoblasts were situated close to the cementum surface in the periodontal ligament and constituted ovoid or round, plump cells with a basophilic cytoplasm. These cells were present singly or in groups of up to 4 cells. The cementocytes were present in the cemental lacunas. In the saline treated rats, a normal thickness of cementum was seen without any observable increase in the thickness of the cementum. In the CsA treated rats, the mean increase in the thickness of cementum was found to be 13.183 μ as compared to saline treated rats (4.733 μ). The difference between these two groups was statistically significant with the *P* value of < 0.0001 [Figure 6].

DISCUSSION

Though the role of cyclosporine A on the individual periodontal tissues has been evaluated in several animal and human studies, the assessment of its effects on all the three periodontal tissues (gingiva, alveolar bone and cementum) in a single experimental study makes it unique from other studies. In the present morphometric and histological analysis on rats, CsA was found to act differentially on all three periodontal target tissues: GO, alveolar bone loss and NC formation, after an experimental period of 45 days. These findings support the observations made by other authors in their addressing this issue.[4-8,12-14]

The CsA-induced GO has been observed in various animal models[4,5] and human clinical studies.[6] In the present morphometric assessment of the drug induced GO model, clinically evident increase in the dimensions of gingiva with significant change in the morphology was observed in the CsA group rats as compared to the saline treated rats, in a 45 day experimental period. Though the exact mechanism is not clear, it has been proposed that the drug acts directly or indirectly on the growth and function of both gingival fibroblasts and collagen fibers through its alteration in various growth factors and cytokines.[15] The potent CsA effect on TGF-β transcription and secretion has been observed *in vitro* in human T lymphocytes, mouse proximal tubular cell lines and tubule-interstitial fibroblasts and *in vivo* in mouse and rat kidneys.[16] It is possible that CsA acts through similar pathway of development and regression of CsA-induced GO during treatment.

There are evidences to show that CsA causes increased bone turnover, with higher resorption than formation, resulting in bone loss.[7,9,10,17] CsA-induced osteopenia has been reported to be associated with an increased incidence of bone fractures.[17] Studies have shown that CsA therapy in rats has also caused increased osteoclasia and decreased bone formation at periodontal sites.[7] However in contrast to these findings, there are reports of significantly less alveolar bone loss and more bone formation in the experimental periodontitis model in rats treated with CsA, biphasic effects of CsA on osteoblast differentiation and bone formation was observed.[15-17] Studies have postulated that CsA exerts its osteopenic effect through the T-cell rather than directly on bone.[9] Other hypothesis may be that, CsA mediates its osteopenic effect by interfering in the cytokine activity on both osteoclasts and osteoblasts at the bone microenvironment; thus, influencing bone remodeling.[17] One recent study suggested that folic acid may have a protective effect on the CsA-induced alveolar bone loss.[10] In the present morphometric and histological study, evaluation of the specimens revealed a significant amount of alveolar bone loss in the CsA group rats as compared to control (saline) group; though, in some areas, there was also an evidence of concomitant formation and resorption of alveolar bone. These results are in agreement with the findings of earlier studies.[7,9,10,17]

Cementum plays a key role in the periodontal attachment apparatus through the gingival and periodontal connective tissue fiber insertion. However in periodontal disease, this attachment apparatus including the cementum is adversely affected owing to the inflammatory process resulting in the suspect integrity and stability of the tooth. Hence, one of the main objectives of periodontal therapy includes the regeneration and reformation of these lost periodontal tissues including the cementum. The present histological study revealed large amount of NC formation mainly in the cervical areas with insertion of newly formed collagen fibers into it but no such changes were seen in the saline group [Figure 5]. The NC is characterized by (1) its large volume (2) irregular outline (3) rather infrequent observations of incremental outlines and (4) presence of globular bodies. Connective tissue adjacent to the newly formed cementum shows densely packed collagen fibers and were seen to be functionally inserting into the newly formed cementum. This finding was in accordance with the observations made in the previous studies.[12-14] However, present study stands different in terms of providing both quantitative and qualitative data of the CsA-induced NC formation.

TGF-β over-production due to CsA may be one of the mechanisms that could explain the greater thickness of cementum formation.[17] Another report demonstrated that CsA may influence both proliferation and differentiation of human periodontal ligament cells, which may play an important role in the homeostasis of periodontal tissue.[1] Frequently, new attachment requires NC formation to replace diseased root surfaces contaminated with the bacterial endotoxins, which are eliminated during periodontal therapy. The formation and regulation of NC in humans is not so clear. The connective tissue matrix of cementum sequesters growth factors such as the TGF-β, fibroblast growth factors and cementum

derived growth factor as well as a battery of other polypeptides, including osteopontin, bone sialoprotein-II and cementum-specific attachment protein, which mediate cell adhesion and spreading. These molecules affect the migration, attachment and proliferation of periodontal cells and their matrix synthesis and more importantly, they manifest cell specificity and tissue specificity among the same cell type. The extracellular matrix of cementum has the potential to regulate the differentiation of precursor cells into cementoblasts. Thus, cementum components are capable of providing informational signals for the recruitment, proliferation and differentiation of periodontal cells and regulate the regeneration of cementum as well as adjacent periodontal components.[11,18]

One of the experimental studies demonstrated that CsA administration leads to the formation of new cementum like islets (NCLI) inside the gingival connective tissue. These islets were located adjacent to blood vessels near the root surfaces. NCLI were found to be engulfed by the multinucleated cells due to their location near the blood vessels.[19] Previous investigations revealed that NC formed during CsA administration is maintained even after suspension of the treatment.[20]

There is a potential for future studies on different doses of CsA on gingiva, alveolar bone and NC.

From this experimental study, it can be concluded that periodontal regeneration, which is an ultimate goal of any periodontal therapy can be achieved through CsA induced cementogenesis. Since systemic administration CsA at the clinical level to achieve cementogenesis and periodontal regeneration will be ethically unwarranted and unacceptable, further animal studies can be conducted to assess the effect of local application of CsA on the planed root surfaces would result in cementogenesis and periodontal regeneration. Hence within the limits of this experimental study, it can be concluded that CsA in a immunosuppressive dose on rats has got its negative effect on gingiva and bone, characterized by GO and alveolar bone loss. However, it has also got one positive effect on cementum where it causes NC formation with the insertion of new collagen fibers. When all these effects of CsA are considered, it can be concluded that CsA has its target on periodontal tissue causing GO, alveolar bone loss and NC formation and this could be a new triad of CsA effects on periodontium.

CONCLUSION

CsA targets the periodontal tissues (gingiva, alveolar bone and cementum) in different pattern. Its role in cementogenesis can be utilized for periodontal regeneration if its local application is testified and verified in the future animal studies.

ACKNOWLEDGEMENTS

We would like to acknowledge the help and cooperation given by Dr. Ahmed Mujib, Professor and Head, Department of Oral Pathology and Microbiology for carrying out the histopathological work for this study.

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How to cite this article: Jayasheela M, Mehta DS. The role of cyclosporine A on the periodontal tissues. Dent Res J 2013;10:802-8.

Source of Support: We would like to thank JJM medical College, Davangere, India for Animal House facility and Bapuji Dental College & Hospital, Davangere, India for the Laboratory facility.
Conflict of Interest: None declared.