

## Original Article

# Proliferative and inductive effects of Cyclosporine a on gingival fibroblast of child and adult

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## ABSTRACT

**Background:** Gingival overgrowth is a serious side-effect that accompanies the use of Cyclosporin A (CsA). Up to 97% of the transplant recipient children, who were submitted to CsA therapy, have been reported to suffer from this side-effect. Several conflicting theories have been proposed to explain the fibroblast's function in CsA-induced gingival overgrowth. The aim of this study is to assess the proliferation of gingival fibroblasts and levels of released cytokines after being exposed to CsA, in both adults and pediatric groups, and to make a comparison between the results of the two groups.

**Materials and Methods:** The adult fibroblast samples were derived from four healthy adults, aged 35 to 42 years and pediatric samples were obtained from four healthy children, age between four and eleven years. Tissue samples were plated in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), Streptomycin and Penicillin. The samples were cultured in 25 cm<sup>2</sup> plates containing 5% CO<sub>2</sub>, and incubated at 37°C. The cells used for all the experiments were at the fourth passage. The concentration of PGE<sub>2</sub>, IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ , and TGF- $\beta$ <sub>1</sub> was determined by the enzyme-linked immunosorbent assay (ELISA) and the proliferation rate was assessed by the MTT assay. Alpha error levels were set as 0.05.

**Results:** CsA stimulated significantly higher levels of IL-6, IL-8 and TGF- $\beta$ <sub>1</sub> in adult gingival fibroblasts than it did in the control group; whereas, the expression of IL-1 $\beta$  and PGE<sub>2</sub> in the fibroblasts exposed to CsA was significantly weaker ( $P < 0.05$ ). The fibroblasts in the two groups did not reveal any noticeable difference in the production of TNF- $\alpha$ . Furthermore, cell proliferation in the CsA group was not significantly higher than that in the control group. No significant differences in cytokines TNF- $\alpha$  and IL-1 $\beta$  were noted between the two groups. The results indicated that CsA stimulated cell proliferation in the pediatric fibroblast cell line. Comparison between the results in the adult and pediatric groups demonstrated that the levels of IL-1 $\beta$ , IL-6, IL-8, and PGE<sub>2</sub> were significantly higher in the pediatric group than in the adult group; however, statistics showed no significant difference in the levels of TNF- $\alpha$  and TGF- $\beta$ <sub>1</sub> and CsA-induced proliferation between these two groups.

**Conclusions:** The mechanism of a CsA-induced fibroblast overgrowth may converge on the steps involving fibroblast proliferation and cytokine network including IL-6, IL-8, IL-1 $\beta$ , TGF- $\beta$ <sub>1</sub>, and PGE<sub>2</sub>, in both adults and pediatrics. As the prevalence and intensity of drug-induced gingival overgrowth is more serious in the pediatrics. As group than in adults, we suggest that more studies be conducted on the pediatric group.

**Key Words:** Cyclosporin, gingival hyperplasia, fibroblasts, Phenytoin

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## INTRODUCTION

Gingival overgrowth is a serious side-effect accompanying the use of Cyclosporin A (CsA) and it is exacerbated by bacterial plaque. This overgrowth hampers normal oral functions such as mastication and speech, restricts oral hygiene, and subsequently,

results in psychological problems.<sup>[1,2]</sup> The incidence of CsA-induced gingival overgrowth is in the range of 25-81% of the patients.<sup>[3]</sup> In addition, up to 97% of the transplant recipient children, who have been submitted to CsA therapy, are reported to suffer from this side-effect.<sup>[4]</sup> Researchers have linked the incidence and severity of gingival overgrowth to several factors such as age, sex, duration of treatment, and dosage of the prescribed CsA.<sup>[5-7]</sup> Cyclosporin A, a cyclic endecapeptide with an immunosuppressive effect, is widely used in the treatment of special autoimmune diseases, and to prevent graft rejection in organ transplant patients.<sup>[8]</sup> Several approaches, such as drug substitution or CsA dose reduction, as well as oral hygiene programs and surgical interventions, have been suggested to treat CsA-induced overgrowth. However, each of these approaches has its own complexities. Reducing the dosage or using alternative drugs is not practical in all situations, and other drugs have their own side-effects, too. On the other hand, surgical intervention is only proposed for cosmetic cases; and finally, oral hygiene programs are only capable of reducing the overgrowth, not inhibiting its development.<sup>[9]</sup>

Imbalances between the synthesis and degradation of components of the extracellular matrix (ECM) cause a deposit of the matrix, which has been suggested as one of the most significant events is gingival growth.<sup>[1,10]</sup> Although the accurate mechanism underlying this process has not been clarified yet, it has been supposed that alternations in gingival fibroblast activity via regulating several cytokines causes gingival enlargement.<sup>[11]</sup> Complex interactions between mediators and growth factors might be responsible for CsA-induced gingival overgrowth. Some studies show that CsA alters the transcription of several cytokines such as the transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1). TGF- $\beta$ 1 has been reported to play a significant role in decreasing the proteolytic activity of gingival fibroblasts in CsA-induced gingival overgrowth.<sup>[12]</sup> TGF- $\beta$ 1 participates in healing fibrosis and synthesizing of CEM components.<sup>[13-16]</sup> Recent studies have shown that CsA raises the amount of TGF- $\beta$ 1 in gingival crevicular fluid (GCF) samples in CsA-treated patients. Even as the same levels of cytokine have been observed in groups with and without gingival overgrowth, TGF- $\beta$ 1 may not be the sole cause of CsA-induced gingival overgrowth.<sup>[17]</sup> Interleukin-6 (IL-6) and TGF- $\beta$ <sub>1</sub> are the cytokines that participate in gingival tissue turnover. Interleukin-6,

which can be released from fibroblasts, affects connective tissue cells via increasing their proliferation and is involved in collagen and glycosaminoglycans production.<sup>[18,19]</sup> Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) are important pro inflammatory cytokines in chronic gingivitis;<sup>[20,21]</sup> however, TNF- $\alpha$  exhibits a different behavior, like growth-stimulating, inhibitory, and self-regulatory properties. It works in a variety of processes; however, all of these processes have not been found yet.<sup>[22]</sup> Interleukin-8 (IL-8) is also an important inflammatory mediator, produced by human gingival fibroblasts, which is strongly chemotactic for polymorphonuclear neutrophils (PMN) and T cells.<sup>[23]</sup> Several studies have reported a positive correlation between the progression of chronic periodontitis and the expression of IL-8 in the gingival tissue.<sup>[24,25]</sup> Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is reported to have pathogenic effects in periodontal disease progression.<sup>[26]</sup> Recent studies have reported that after CsA therapy, the severity and prevalence of gingival overgrowth are notably higher in the pediatric group than in the adult group.<sup>[4,27]</sup> Their vulnerability to this side-effect may be related to the susceptible immature fibroblasts or to the effect of androgen metabolism.<sup>[28,29]</sup> Although some studies have reported that CsA promotes gingival fibroblast proliferation, other studies have reported a decrease or even no changes in proliferation.<sup>[12,30,31]</sup>

Several conflicting theories have been proposed to explain the fibroblast's function in CsA-induced gingival overgrowth. Moreover, to our knowledge, no studies have investigated the correlation between gingival overgrowth and the levels of released cytokines of fibroblasts induced by CsA in the pediatric group. The aim of this study was to assess the proliferation of gingival fibroblasts and levels of released cytokines after being exposed to CsA in both the adult and pediatric groups, and to make a comparison between the results of the two groups.

## MATERIALS AND METHODS

### Surgical process

The adult fibroblast samples were derived from four healthy adults who were submitted to crown lengthening surgery. The age range was from 35 to 42 years. In addition, the pediatric samples were obtained from four healthy children, age range from four to eleven years, during a procedure of impacted tooth extraction. There were no signs of periodontitis

in both the children and adults. In both cases, a fragment of excess tissue was removed under local anesthesia at the moment of surgery. All the persons were in good health, without any systemic diseases. Smokers, pregnant women, and any drug users were excluded to avoid any probable confounding factors. Informed consent was obtained from each donor prior to taking of samples, and the experimental protocol was approved by the Ethics Committee in the Shahid Beheshti Medical University.

### Cell culture

The tissue samples were plated in Dulbecco's Modified Eagle's Medium (DMEM; Biochrom AG, Berlin, Germany) containing 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), streptomycin (50 U/ml), and penicillin (50 U/ml). The samples were cultured in 25 cm<sup>2</sup> plates, and incubated at 37°C atmosphere containing 5% CO<sub>2</sub>. The samples were regularly controlled for any contamination and cell growth, and then they were fed with fresh medium, if necessary. When the fibroblasts grew out from the explants, they were trypsinized and shifted to the next flasks (Nunc, Copenhagen, Denmark) for a secondary culture. The cells used for all the experiments were at the fourth passage.

### Enzyme-linked immunosorbent assay

Gingival fibroblasts were seeded into 24-well plates (Nunc, Copenhagen, Denmark) at a density of  $60 \times 10^3$  cells/well. The wells were divided into control and experimental groups. After 48 hours, CsA (Sigma-Aldrich, St. Louis, MO, USA) (500 ng/ml) was added to the experimental wells. The samples were then incubated at 37°C in 95% humidified atmosphere containing CO<sub>2</sub>. In order to assess the amount of inflammatory mediators produced by the gingival fibroblasts, the supernatant fluid of each control and experimental well was collected. The concentration of PGE<sub>2</sub>, IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ , and TGF- $\beta$ <sub>1</sub> was determined by the Enzyme-Linked Immunosorbent assay (ELISA), using the ELISA kits, according to the manufacturer's instructions (R and D systems, Minneapolis, MN, USA).

### MTT assay

The MTT assay was performed according to the manufacturer's instructions. The gingival fibroblasts were seeded into 96-well plates (Nunc, Copenhagen, Denmark) at a density of  $5 \times 10^3$  cells/well, and were cultured in a 200 cc

medium. After a 48-hour incubation period, the samples were divided into control and experimental groups. CsA (Sigma-Aldrich, St. Louis, MO, USA) (500 ng/ml) was added to the experimental wells. After 48 hours of incubation, the medium was replaced with 100 cc of a fresh medium containing a 0.5 mg/ml solution of tetrazolium salt 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT; Merk, Darmstadt, Germany) in phosphate-buffer saline (PBS; Sigma-Aldrich, St. Louis, MO, USA). The cells were then incubated at 37°C in 95% humidified atmosphere containing CO<sub>2</sub> for four hours. Dimethyl sulfoxide (DMSO; Merk, Darmstadt, Germany) was used to dissolve the crystals of formazan produced by the living cells. The quantity of formazan was then read at a wavelength of 570 nm with an ELISA plate reader (Statfax, Florida, USA) according to the manufacturer's instructions. The optical density was directly proportional to the number of living cells.

### Statistical analysis

The data released by ELISA were then tested by the Kolmogorov Smirnov test, to evaluate the normal distribution of adult and pediatric gingival cell population. The analysis of variance (ANOVA) and Tukey's Honest Significant Difference (HSD) multiple comparison tests were used for statistical evaluations. Comparisons between the two groups were performed using the student's *t*-test. All calculations were performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). Less than 0.05 alpha error levels were considered statistically significant.

## RESULTS

Due to the natural constraints in the primary cell culture, several pediatric samples were ruined in the experiment; hence, 24 adult samples and 18 pediatric samples were obtained in total.

### Effect of cyclosporin a on adult gingival fibroblasts

Cyclosporin A stimulated significantly higher levels of IL-6, IL-8, and TGF- $\beta$ <sub>1</sub> in adult gingival fibroblasts than it did in the control group; whereas, the expression of IL-1 $\beta$  and PGE<sub>2</sub> in the fibroblasts exposed to CsA, was significantly weaker ( $P < 0.05$ ). Fibroblasts in the two groups did not reveal any noticeable difference in the production of TNF- $\alpha$ . Furthermore, cell proliferation in the CsA group was not significantly higher than that in the control group [Table 1].

**Table 1: The expression of released IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ , TNF- $\beta$ , PGE $_2$  and the extent of proliferation in adult and pediatric fibroblasts in both the control group and the CsA-treated group (Mean $\pm$ sd)**

Mediators	Control	CsA
IL-1 $\beta$ ** (Pg/ml)		
Adult*	4.8 $\pm$ 0.2	4.18 $\pm$ 0.17
Pediatric*	4.63 $\pm$ 0.21	4.55 $\pm$ 0.33
IL-6** (Pg/ml)		
Adult*	95.73 $\pm$ 14.77	137.41 $\pm$ 12.73
Pediatric*	196.86 $\pm$ 4.99	244.43 $\pm$ 21.16
IL-8** (Pg/ml)		
Adult*	114.24 $\pm$ 12.09	195.66 $\pm$ 32.57
Pediatric*	609.71 $\pm$ 343.70	1780.58 $\pm$ 988.40
TGF- $\beta$ <sub>1</sub> (Pg/ml)		
Adult*	9.23 $\pm$ 7.52	29.01 $\pm$ 6.35
Pediatric*	17.86 $\pm$ 2.82	33.4 $\pm$ 4.79
PGE $_2$ * (Pg/ml)		
Adult*	559.99 $\pm$ 37.85	432.28 $\pm$ 12.64
Pediatric*	659.87 $\pm$ 5.26	618.33 $\pm$ 15.32
Proliferation		
Adult	0.14 $\pm$ 0.12	0.18 $\pm$ 0.08
Pediatric*	0.07 $\pm$ 0.06	0.13 $\pm$ 0.06

\*Significant difference between CsA and control group ( $p < 0.05$ ) \*\*Significant difference between CsA adult group and CsA pediatric group ( $p < 0.05$ ) IL: Interleukin; TNF: Tumor necrosis factor; TGF: Transforming growth factor; PGE $_2$ : Prostaglandin E $_2$

### Effect of cyclosporin a on pediatric gingival fibroblasts

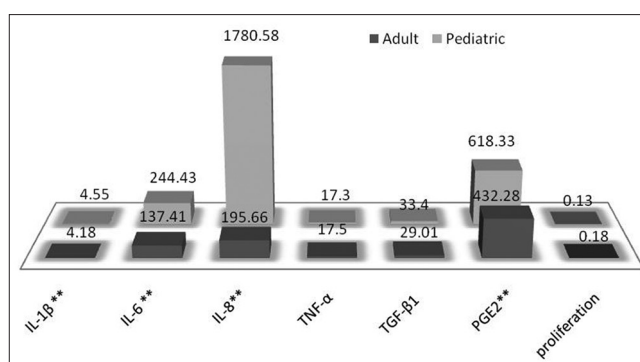
In pediatric gingival fibroblasts, the expression of IL-6, IL-8, and TGF- $\beta$ <sub>1</sub> was significantly enhanced by CsA. In contrast, the levels of PGE $_2$  were significantly lower than those in the control group. No significant difference in cytokines TNF- $\alpha$  and IL-1 $\beta$  were noted between the two groups. The results indicated that CsA stimulated cell proliferation in the pediatric fibroblast cell line [Table 1].

### Comparison between the adult and pediatric groups

Comparison between the results of the adult and pediatric groups demonstrated that the levels of IL-1 $\beta$ , IL-6, IL-8, and PGE $_2$  were significantly higher in the pediatric group than in the adult group; however, statistics showed no significant difference in the levels of TNF- $\alpha$  and TGF- $\beta$ <sub>1</sub> and CsA-induced proliferation between these two groups [Table 1, Figure 1].

## DISCUSSION

The results of this study demonstrated that CsA could affect the levels of IL-6, IL-8, TGF- $\beta$ <sub>1</sub>, IL-1 $\beta$ , and PGE $_2$ . Moreover, CsA seemed not to affect



**Figure 1: Comparison of effect of CsA on the released IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ , TGF- $\beta$ <sub>1</sub>, and PGE $_2$  (Pg / ml), and proliferation between adult and pediatric fibroblasts**

TNF- $\alpha$  production in the fibroblasts. These findings were obtained from ELISA, in which a 500 ng/ml concentration of CsA was used, according to previous studies.<sup>[30,32]</sup> The Micro-culture Tetrazolium Assay (MTA) was also method was also used to evaluate cell proliferation in fibroblasts, which resulted in the cell proliferation in CsA-induced fibroblasts being significantly higher than in the normal fibroblasts.

The levels of IL-6 in CsA-induced gingival fibroblasts were found to be elevated in both the adult and pediatric groups, in comparison to those in the normal fibroblasts. Our results are in agreement with some previous reports that have also reported such an increase.<sup>[31,33]</sup> Although it has been reported that NF- $\kappa$ B may trigger IL-6 gene expression,<sup>[34]</sup> this perceived increase in IL-6 levels may be attributed to the prevalence of the transcriptional regulator NF- $\kappa$ B. It has been reported that CsA can cause a rise in intercellular Ca<sup>++</sup>,<sup>[35]</sup> which in turn, activates NF- $\kappa$ B.<sup>[36]</sup> Our results stand in contrast to the study by Yoshimura, *et al.*,<sup>[37]</sup> who have reported a reduction in the levels of IL-6. However, their study was performed on peripheral blood mononuclear cells rather than on gingival fibroblasts.

In our study, we perceived that CsA triggered the production of IL-8 in gingival fibroblasts, which was in agreement with a previous report by Suzuki, *et al.*<sup>[38]</sup> Also, Leonardi *et al.*<sup>[39]</sup> have shown that CsA-induced conjunctival fibroblasts could enhance the production of IL-8 in a dose-dependent matter; a phenomenon that can be justified by the toxic activity of high doses of CsA.<sup>[39]</sup> Considering the fact that cytokine IL-8 triggered chemotactic activity in inflammatory cells, one might conclude that the drug-induced enhancement of IL-8 production in gingival fibroblasts took part in the recruitment of



mononuclear cells in the gingival tissue during CsA medication.<sup>[23]</sup>

In agreement with the previous studies, our results indicated that CsA could increase the levels of TGF- $\beta$  in gingival fibroblasts.<sup>[40,41]</sup> Chae, *et al.*<sup>[42]</sup> suggested that TGF- $\beta$  contributed to CsA-induced secretion of IL-6. If this was true, the elevated level of IL-6 in our study might be partly stimulated by the perceived increase in TGF- $\beta$ .

In comparison to normal fibroblasts, CsA-induced fibroblasts produced lower levels of IL-1 $\beta$ . In the pediatric group, however, this reduction did not reach a significant difference. Our results are in a line with some other studies that have also reported such a decline.<sup>[33,43]</sup>

In our study, CsA-induced fibroblasts demonstrated no significant difference in TNF- $\alpha$  production compared to the normal fibroblasts. Contrary to these results, Subramani, *et al.*<sup>[22]</sup> reported an elevated level of TNF- $\alpha$  after CsA induction. In that study, the samples were obtained from the overgrown gingival tissues of CsA-treated patients. Although gingival overgrowth was usually accompanied with plaque-induced inflammation, which was seen to have an increasing effect on the secretion of inflammatory cytokines and mediators,<sup>[44]</sup> the reported increase in the TNF- $\alpha$  level in that study could be attributed to the possible inflammation of the tissues. Another possible explanation for this disagreement might be that Subramani, *et al.*'s study was performed on gingival tissue, in which cells other than fibroblasts could have been responsible for increasing the level of TNF- $\alpha$ .

The levels of PGE<sub>2</sub> in CsA-induced gingival fibroblasts were significantly lower than those in the normal fibroblasts, a phenomenon that confirmed one previous report.<sup>[33]</sup> However, this result was in contrast to the study by Windom *et al.*,<sup>[45]</sup> who reported that induction of CsA at 100 ng/ml did not significantly alter PGE<sub>2</sub> secretion. One possible explanation might be that the influence of CsA on fibroblasts was dose-dependent.

In an attempt to investigate the effects of cytokines on gingival overgrowth, our results indicate that changes in the levels of experimented cytokines after CsA induction may induce gingival overgrowth. It has been reported that IL-6 reduces tissue breakdown through stimulating tissue inhibitor of metalloproteinase (TIMP) expression, resulting in accumulation of

the extracellular matrix. Opposed to IL-6, IL-1 $\beta$  is shown to induce tissue breakdown by triggering metalloproteinase secretion.<sup>[33]</sup> Thus, our results indicate an increase in IL-6 levels and a decrease in IL-1 $\beta$ , suggesting that both these cytokines contribute to CsA gingival overgrowth.

TGF- $\beta$ <sub>1</sub> contributes to gingival overgrowth not only by direct influence in cell proliferation, but also by accumulating extracellular matrix in several ways. First, it has an influence on collagen metabolism such that it inhibits the collagen degradation process.<sup>[46]</sup> Second, TGF-1 $\beta$  can upregulate gene expression for extracellular matrix components, especially collagenous proteins.<sup>[47]</sup> Finally, it has been suggested that TGF- $\beta$ <sub>1</sub> stimulates growth factors such as the platelet-derived growth factor (PDGF).<sup>[47]</sup> These reports, in addition to our findings indicate that the CsA-induced increase in TGF- $\beta$ <sub>1</sub> contributes partly to gingival overgrowth. The other cytokine, IL-8, is known to have proangiogenic activity.<sup>[48]</sup> Thus, the perceived CsA-induced gingival overgrowth may be related to the enhanced production of IL-8 and increase in the number of microvessels.<sup>[49]</sup> Furthermore, PGE<sub>2</sub> is suggested to be associated with the destruction of connective tissue.<sup>[50]</sup> Therefore, the perceived decline in the levels of this cytokine, once again, suggests that PGE<sub>2</sub> plays a role in gingival overgrowth.

In our study, cyclosporin induced cell proliferation has increased in both groups, although in the adult group it has not reached a significant difference. The reported findings on the effects of CsA induction on cell proliferation are inconsistent and controversial.<sup>[12,30,31]</sup> It has been suggested that the susceptibility of cells to CsA ranges from 8 to 70% and it is possible that the cells may or may not bond to CsA.<sup>[18]</sup> Several explanations can justify such contradictory results. For example, as individuals with variant genetic predispositions demonstrate variant susceptibilities to CsA-induced gingival overgrowth, it is likely that studies using different samples result in different conclusions.<sup>[1]</sup> Moreover, the phenotypic heterogeneity, which has been known to exist between different cells of a single fibroblast culture, may cause the fibroblasts to exhibit different responses to CsA induction.<sup>[51,52]</sup>

In this study, for the first time, CsA-induced levels of cytokines and cell proliferation were assessed in pediatric fibroblasts. Although the trend of alterations

was similar to that in adult fibroblasts, some interesting differences were also perceived. In most cases, the levels of CsA induced-cytokines were higher in the pediatric fibroblasts than in adults. Cell proliferation was also higher in the pediatric group. These differences could be attributed to susceptible immature fibroblasts, to the effects of androgen metabolism, or to other unknown reasons.<sup>[28,29]</sup> Although, one recent study reported that plaque accumulation was the only factor involved in gingival overgrowth in the pediatric group,<sup>[53]</sup> our results indicated that this overgrowth was also dependent on the fibroblast's activity.

## CONCLUSION

The mechanism of CsA-induced fibroblast overgrowth may converge on the steps involving fibroblast proliferation and cytokine network including IL-6, IL-8, IL-1 $\beta$ , TGF- $\beta$ <sub>1</sub>, and PGE<sub>2</sub>, in both adults and pediatrics. Even as the prevalence and intensity of drug-induced gingival overgrowth are more serious in pediatrics than in adults, we suggest that more studies be conducted on the pediatric group.

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