Effect of Cyclosporin A and Angiotensin II on cytosolic calcium levels in primary human gingival fibroblasts

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ABSTRACT

Background: To evaluate the effect of Cyclosporin A (CsA) and angiotensin II (Ang II) on cytosolic calcium levels in cultured human gingival fibroblasts (HGFs).

Materials and Methods: Healthy gingival samples from six volunteers were obtained, and primary HGFs were cultured. Cell viability and proliferation assay were performed to identify the ideal concentrations of CsA and Ang II. Cytosolic calcium levels in cultured gingival fibroblasts treated with CsA and Ang II were studied using colorimetric assay, confocal and fluorescence imaging. Statistical analyses were done using SPSS software and GraphPad Prism.

Results: Higher levels of cytosolic levels were evident in cells treated with CsA and Ang II when compared to control group and was statistically significant (P < 0.05) in both colorimetric assay and confocal imaging. Fluorescent images of the cultured HGFs revealed the same.

Conclusion: Thus calcium being a key player in major cellular functions, plays a major role in the pathogenesis of drug-induced gingival overgrowth.

Key Words: Angiotensin II, calcium, Cyclosporin A, gingival overgrowth

INTRODUCTION

Drug-induced gingival overgrowth (DIGO) is one of the predominant side effects of certain systemically administered drugs such as phenytoin, nifedipine and cyclosporin A (CsA). Several theories have been put forth by researchers to elucidate the pathogenesis of DIGO. Risk factors such as age, poor oral hygiene, genetic predisposition, dosage, duration of drug intake, and gingival inflammation influence the relationship between the drugs and gingival tissue.[1] Hormones are known to play a vital role in DIGO. Prior studies suggest that receptors for testosterone[2] and cortisol[3] are increased in DIGO. Recently, the role of renin–angiotensin system and endothelin 1 (ET 1) in the pathogenesis of DIGO has been widely researched.[4,5]

Angiotensin II (Ang II) being the effector peptide of renin–angiotensin system, elicits series of complex highly regulated cascades of intracellular signal transduction that leads to short-term vascular effects such as contraction and long-term biological effects such as cell growth, migration, extracellular matrix deposition, and inflammation. Apart from playing a key role in regulating local and systemic

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hemodynamics, it has been considered as a true cytokine with regards to renal pathology.\[6\]

Ang II has been known to upregulate chemotactic factors such as monocyte chemotactic protein-1 which could in turn stimulate fibrosis. It is noteworthy that Ang II also affects the intracellular calcium ($Ca^{2+}$) levels.\[7\] An interesting fact about the relationship between calcium and the pathogenesis of DIGO is that almost all the drugs implicated in the gingival overgrowth are known to be calcium channel blockers. Calcium functions as a key modulator in various vital cellular functions. Literature review shows that the effect of CsA on calcium metabolism varies in different types of cells. Classically, CsA was known to block the calcium channel blockers. However, there are results from other studies which prove otherwise, where the intracellular calcium in cells treated by CsA showed increased cytosolic calcium levels.\[8,9\] Hence, this study was performed to assess the effect of CsA and Ang II on intracellular calcium levels of human gingival fibroblasts (HGFs).

MATERIALS AND METHODS

Ethical clearance
This study was approved by the Institutional Ethics Committee at Sri Ramachandra University, Chennai, India. Gingival tissue biopsy samples were obtained from six systemically healthy volunteers undergoing crown lengthening procedure at Department of Periodontology, Faculty of Dental Sciences, Sri Ramachandra University, Chennai. After describing the study procedure, informed consent was obtained from all volunteers before sample collection. Volunteers who were included in the study were not under any systemic medications so as to avoid confounding effects on the results.

Details of chemicals, reagents, and instruments used
Low glucose containing Dulbecco’s Modified Eagle Medium (DMEM) and trypsin were purchased from HiMedia (Mumbai, Maharashtra, India). EU certified fetal bovine serum and antibiotics were procured from Gibco (Waltham, MA, USA). Cells were counted with tryptan blue dye from Sigma-Aldrich (Bengaluru, Karnataka, India), all plasticware was purchased from Nunc Nalgene (Rochester, NY, USA). Calcium analysis was performed using a calcium green 1 AM from Invitrogen Bioservices (Bengaluru, Karnataka, India). All other chemicals of an analytical (research) grade were purchased from Sigma-Aldrich. EnSpire Multimode Plate Reader from Perkin Elmer (Waltham, Massachusetts, USA) was used to quantify fluorescence intensity level. A Nikon TE2000 Eclipse inverted fluorescence microscope with a CCD camera and ImagePro Software (Melville, NY, USA) and A Zeiss LSM 510 confocal microscope (Carl Zeiss, Germany) was used for fluorescence and confocal imaging studies respectively.

Sample collection and culturing of human gingival fibroblasts
The collected gingival samples were preserved in transport media containing 1X phosphate-buffered solution and antibiotics (penicillin - 100 IU/ml, streptomycin - 100 μg/ml, amphotericin B - 100 μg/ml, gentamicin-200 μg/ml, and ciprofloxacin-200 μg/ml) until they were processed. HGFs were cultured using enzymatic digestion method\[10\] using. Monolayer cultures were seeded in culture plates with complete growth essential medium (DMEM low glucose supplemented with 10% FBS and antibiotics) at 37°C and under 5% CO$_2$ in an incubator. The cultures were observed every day [Figure 1], and the medium was replenished once in 2 days. Once the HGF attained 80–90% confluence, they were trypsinized and used for the assays. In this study, we have used HGF at Passage P5 to P8.

Cell viability and proliferation assay
To determine the viability and cell proliferation rate of HGF, live-dead cell staining (acridine orange/ethidium bromide [AO/EtBr]), and colorimetric tetrazolium salt 3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) test were performed.

Cell viability assay
Cultured HGF cells were treated with different concentrations of CsA - 10, 25, and 50 μM and Ang II - 100 nM, 1 μM, 10 μM, and 100 nM for 24 h. AO/EtBr (1 μg/ml) is a combination of AO and EtBr used to stain the cells treated with various concentrations of CsA and Ang II indicated above. AO stains both cytoplasmic and nuclei staining of live cells (green) whereas EtBr stains only the dead cell nuclei (red). After staining with AO/EtBr, HGF cells in all the 3 groups (control, CsA, and Ang II) were imaged using fluorescence microscopy.\[7,11\]

Cell proliferation assay
Cultured HGF cells were treated with different concentrations of CsA - 10, 25, and 50 μM and Ang II - 100 nM, 1 μM, 10 μM, and 100 nM for 24 h. AO/EtBr (1 μg/ml) is a combination of AO and EtBr used to stain the cells treated with various concentrations of CsA and Ang II indicated above. AO stains both cytoplasmic and nuclei staining of live cells (green) whereas EtBr stains only the dead cell nuclei (red). After staining with AO/EtBr, HGF cells in all the 3 groups (control, CsA, and Ang II) were imaged using fluorescence microscopy.\[7,11\]
concentrations of CsA (1, 5, 10, 25 and 50 µM) and Ang II (100 nM, 1 µM, 1 µM, and 100 µM). After the incubation time of 24 h, the MTT test was performed by a previously described method. The optical density of each well was measured using a multimode reader at 570 nm reference wavelength, and 50% inhibition of viability (IC 50) was calculated for both CsA and Ang II drug, respectively. All the experiments were repeated for the same concentration in triplicates for each HGF cell sample.

**Intracellular calcium intensity measurement by multimode plate reader**

HGF cultured from six individual volunteer samples (5–8 passages) were seeded in a 96-well plate. Once the wells attained 80–90% confluence, the cells were incubated with 50 µl of 25 µM concentration of calcium green 1 AM for 45 min at 37 ºC in dark. The solution containing the dye was discarded, and 100 µl of 25µM of CsA and 100 nM of Ang II were added to the respective wells, and calcium intensity was immediately measured using Perkin-Elmer EnSpire Multimode Plate Reader. The excitation and emission wavelength was set at 506 and 531 nm, respectively. The calcium intensity in HGF was measured in a time-dependent manner varying from 10 to 100 s immediately after addition of both the compounds (CsA and Ang II) along with the control sample.

**Intracellular calcium imaging by fluorescence microscopy**

The HGF cells were seeded in a 96-well plate. Once the cells attained 80–90% confluence, they were incubated with 25 µM of calcium green 1 AM for 45 min. Just before imaging, 100 µl of 25 µM of CsA and 100 nM of Ang II were added to the wells. Nuclei were counterstained with 4, 6diamidino-2-phenylindole (DAPI). The cells were observed using a Nikon TE2000 Eclipse inverted fluorescence microscope and images were captured using a CCD camera and ImagePro Software.

**Intracellular calcium imaging by confocal microscopy**

HGF cells were grown on sterile glass coverslips. Once the cells were 80–90% confluent, live staining of intracellular calcium was done by incubating the cells with 25 µM of calcium green 1 AM for 45 min. Just before imaging, 100 µl of 25µM of CsA and 100 µM of Ang II were added into the appropriate coverslips. Confocal images were obtained using a Zeiss LSM 510 Confocal Microscope (Carl Zeiss, Germany).

**Statistical analysis**

All experimental data are represented as the mean ± standard deviation. Unpaired t-test was used to analyze the MTT results. With regard to calorimetric assay results, one-way ANOVA and post hoc tests with multiple
comparisons (Tukey’s Honestly Significant Difference) were done to analyze the data statistically using SPSS 16.O (Kacharanahalli, Bangalore, India), Bengaluru, India, whereas Bonferroni posttests using Graphpad prism 6; Graphpad software Inc, (La jolla, California, USA) were used to analyze data from confocal imaging. P < 0.05 is set as statistically significant, P < 0.01 is set as highly significant and P < 0.001 is set as very highly significant.

RESULTS

Cell viability and proliferation assay of Cyclosporin A and Angiotensin II-induced human gingival fibroblasts cells
Preliminary effects of CsA and Ang II on HGF cells showed dose-dependent cytotoxicity (i.e., increased cytotoxicity with increasing concentrations of CsA [10, 25, and 50 µM] and Ang II [100 nM, 1 µM, and 10 µM]). The corresponding results showed more than 50% of viable cells when compared to control [Figure 2a (i) and b (i)].

HGF proliferation was determined by MTT assay after 24 h incubation with CsA and Ang II. We observed most satisfactory results. With increasing dose of CsA (50 µM) and Ang II (100 µM) per culture [Figure 2a (ii) and b (ii)], HGF cells showed significantly reduced cell growth compared with the minimal concentration of CsA (10 µM) and Ang II (1 µM). The required concentration for 50% inhibition of cell growth by CsA (23.25 µM) and Ang II (59.07 µM) was extrapolated from the dose response curve using GraphPad Prism and with P < 0.01 [Figure 2a (iii) and b (iii)].

Intracellular calcium imaging by fluorescence imaging
Live calcium staining was done by incubating HGF with calcium green 1 AM for 1 h and later nuclei were counterstained with DAPI (blue). Just before imaging, CsA (25 µM) and Ang II (100 nM) were added in their respective wells. Imaging was done using a Nikon TE Eclipse 2000 Immunofluorescence Microscopy under ×10 magnification. Maximum calcium staining was observed in CsA followed by Ang II [Figure 3a]. However, control sample exhibited the least intensity [Figure 3a].

Colorimetric assay
Time-dependent calcium intensity measurement from 10 to 100 s was done in all the three groups (control, CsA, and Ang II) by incubating the cells with calcium green 1 AM using a multimode plate reader. CsA group showed maximum calcium intensity followed by Ang II. A mild peak in calcium intensity occurred.

Figure 2: Human gingival fibroblast cells were incubated with Cyclosporin A (a - i, ii, iii) and Angiotensin II (b - i, ii, iii). After 24 h, incubation cell viability (acridine orange/ethidium bromide) and 3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide assay were analyzed. Data were expressed as nonlinear regression analysis of the concentration-response. CsA: Cyclosporin A; Ang II: Angiotensin II.
around 20–30 s in CsA and Ang II, following which there was a fall followed by a sustained plateau in all the groups [Figure 3b]. At all the time points (10–100 s), there was a statistically significant difference in calcium intensity between control and CsA (highly significant, $P < 0.010$). Furthermore, there was a statistically significant difference between control and Ang II (significant, $P < 0.05$). However, there was no statistically significant difference in calcium intensity between CsA and Ang II.

**Intracellular calcium imaging by confocal microscopy**

Three HGF samples were cultured and grown on coverslips. Maximum calcium staining was observed in CsA followed by Ang II and least staining was observed in control sample [Figure 4a]. There was statistically significant increase in fluorescence intensity in CsA and Ang II groups when compared to control ($P < 0.001$) as denoted in Figure 4b. For further analysis, a particular cell in the field of focus was selected, and the intensity was measured and a graphical representation was generated which showed similar results [Figure 5].

**DISCUSSION**

The pathogenesis of DIGO has been extensively studied. Results of which led to the concept of
overgrowth being attributed to increase in synthesis of extracellular matrix rather than increase in cellular proliferation. This is possibly due to the interplay between increased synthesis and impaired degradation of extracellular matrix.\textsuperscript{[13,15,16]}

Drugs such as CsA that induce gingival overgrowth possess calcium channel blocking effect pharmacologically and it is observed to increase intracellular calcium. In a similar way, in CsA-induced renal fibrosis, the drug influences the cell through Ang II. Animal studies have demonstrated the influence of CsA on Ang II in gingiva. This study was carried out to observe if CsA and Ang II have a similar effect on HGF intracellular calcium. In our pursuit, we demonstrated a significant increase in intracellular calcium in HGFs when treated with CsA and Ang II, which was statistically significant ($P < 0.01$) when compared with the controls. Our results were in accordance with the results of a previous study\textsuperscript{[8]} in which the calcium levels in freshly cultured fibroblasts from CsA-induced overgrowth samples were found to be higher than the control group and nifedipine drug group. On the contrary, this study focused on the effect in normal fibroblasts with the addition of CsA. Another study showed the influence of CsA on intracellular calcium in porcine proximal tubule-like cell line LLCPK1 treated with thapsigargin\textsuperscript{[9]} and found that CsA potentiated the increase in cytosolic calcium induced by thapsigargin. Lo Russo \textit{et al.}\textsuperscript{[17]} reported that maximal CsA-induced $\text{Ca}^{2+}$ potentiation occurred in vascular smooth muscle cells.

Recently, a locally operating renin–angiotensin system has been identified in the gingiva and Ang II has been known to increase matrix proteins. Ang II has been implicated in various physiological functions at a molecular level such as proliferation and hypertrophy and it is also known as a morphogenic cytokine. In a study,\textsuperscript{[18]} it was noted that nifedipine-induced expression of Ang II and ET 1 in gingival fibroblasts by acting through AT1 and ETA receptors, respectively. In our laboratory, we also observed that there was an increased Ang II concentration in nifedipine-induced gingival overgrowth.\textsuperscript{[19]} Arora \textit{et al.}\textsuperscript{[20]} in their study on fura-2-loaded rat cells found a steep and rapid increase in intracellular calcium which returned to baseline levels within 50 s in the normal calcium-containing medium. In this study, similar to Arora \textit{et al.}, there was an increase in baseline intracellular calcium levels in CsA-treated HGF in normal calcium-containing medium, but the increase sustained as a plateau (for nearly 100 s) contrary to the peak obtained. This could be attributed to the different cell type, species and the technique that was experimented in this study. This could be due to the few cells in which CsA increases intracellular calcium without blocking the membrane channels. Oguro and Tsuchikawa\textsuperscript{[7]} studied intracellular calcium levels in cultured HGF when treated with nifedipine and Ang II. According to Oguro and Tsuchikawa, Ang II caused increased cytosolic calcium individually and when added along with nifedipine. This study found a similar increase in intracellular calcium when

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Confocal imaging analysis study shown that cyclosporin A and angiotensin II-treated human gingival fibroblast cells stained with calcium green 1 AM and respective graphical representation shown the fluorescence intensity levels (a-c). CsA: Cyclosporin A; Ang II: Angiotensin II.}
\end{figure}
HGF was treated with CsA or Ang II independently confirming the individual effect of each agent.

According to our study results, the dynamic time-dependent calcium intensity curve showed a mild initial spike followed by a sustained plateau when exposed to Ang II. Ang II typically mediates a biphasic Ca\(^{2+}\) response comprising a rapid early transient phase and a sustained plateau phase as we have explained above. The initial transient Ca\(^{2+}\) phase is generated primarily by inositol 1, 4,5-triphosphate-induced mobilization of calcium from intracellular calcium stores.\(^{[21]}\) The second sustained Ca\(^{2+}\) phase, which appears to contribute to the sustained Ang II-induced vasoconstriction, is dependent on external Ca\(^{2+}\), and it is the result of transmembrane Ca\(^{2+}\) influx.\(^{[22]}\) The initial spike of calcium intensity in Ang II-treated HGF can be attributed to the release of calcium from intracellular stores and the later sustained phase manifesting as a plateau could be due to the steady entry of extracellular calcium into the cells through the ion channels. The increase in calcium intensity in CsA and Ang II group which was observed by multimode plate reader method was further confirmed by live intracellular calcium staining observed through the fluorescence and confocal imaging which also showed similar finding. The influence of other drugs such as verapamil which cause gingival overgrowth on cytosolic calcium has also been studied and showed similar results.\(^{[23]}\)

Sobral et al. suggested that CsA-induced expression of transforming growth factor β (TGF-β) in HGFs.\(^{[24]}\) According to Wu et al., connective tissue growth factor (CTGF) expression was known to be increased in HGFs on exposure to CsA.\(^{[25]}\) Ang II is also known to stimulate transcription and synthesis of TGF-β. It is well known that TGF-β and CTGF are known to raise intracellular calcium levels.\(^{[25,26]}\) CsA as well as Ang II are known to upregulate the growth factors; this could be one possible mechanism through which cytoplasmic calcium may be increased.

One of the major consequences of elevated cytosolic calcium could be the activation of c-Jun and c-Fos. C-Jun in combination with c-Fos, forms the activator protein-1, an early response transcription factor. Cruzalegui et al.\(^{[27]}\) identified that c-Jun acts as a calcium-regulated transcriptional activator and also plays a role in the control of coactivator function (i.e., recruitment and stimulation of cAMP-responsive element – binding protein). Earlier a study from our laboratory has shown Ang II in combination with Cyclosporin A modulating the expression of c-Jun and c-Fos in HGF. CsA seems to increase the intracellular calcium, which could cause oxidative stress and endoplasmic reticulum stress. Increased intracellular calcium has been cited as one of the causes for CsA-induced nephrotoxicity, hypertension, and may be gingival overgrowth. The results of this study and our other communicated studies on ROS generation by HGFs treated with CsA could give a different dimension to the pathogenesis of gingival overgrowth and may lead the way to more preventive treatment strategies for this condition. However, the limitation of this study is that it did not assess the synergistic effects of CsA and Ang II on intracellular calcium levels. In conclusion, this present study clearly demonstrates that increase in intracellular calcium takes place in HGFs following CsA or Ang II treatment. Research studies in the future can be directed toward studying the effect of increased intracellular calcium on the HGF metabolism.

CONCLUSION

CsA is a medically important drug, which is used in organ transplant conditions. Apart from its other well-known side effects, it is also known to cause enlargement of gingiva which is termed as CsA-induced gingival overgrowth. Among the various pathways put forth in regard to the pathogenesis of DIGO, RAS, and its role in calcium homeostasis has been studied recently. Thus, we sought to analyze the effect of CsA and Ang II on cytosolic calcium levels in cultured HGFs. The results of our study showed an increase in cytosolic calcium levels when HGF were treated with CsA and Ang II. This mechanism could plausibly upregulate certain important transcription factors such as c-Jun which is implicated in the pathogenesis of DIGO. Thus, it is noteworthy to research more on this area and bring out therapeutic strategies accordingly.

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Conflicts of interest
The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.
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