

# Increased Advanced Oxidation Protein Products Generation by Cyclosporine-A and Angiotensin II in Human Gingival Fibroblasts – Ex-vivo Study

SURESH RANGA RAO<sup>1</sup>, RAJASEKARAN SUBBARAYAN<sup>2</sup>, SUPRAJA AJITKUMAR<sup>3</sup>, DINESH MURUGAN GIRIJA<sup>4</sup>

## ABSTRACT

**Introduction:** Cyclosporin-A (CsA), an immunosuppressant, induces renal fibrosis and Renin Angiotensin System (RAS) is known to play a major role. CsA has the potential to increase the oxidative stress; specifically through the Advanced Oxidation Protein Products (AOPP) which could possibly stimulate fibrosis. A similar type of pathology occurs even in the gingiva known as CsA Induced Gingival Overgrowth (CIGO).

**Aim:** This study was undertaken to estimate the AOPP generation by Human Gingival Fibroblasts (HGF) under the influence of CsA and Angiotensin II (Ang II).

**Materials and Methods:** Six healthy gingival tissue samples

were obtained during crown lengthening procedure and primary HGF were cultured using enzymatic digestion method. The ideal non-cytotoxic concentrations of CsA and Ang II were identified using cytotoxicity assay. Later, HGF were incubated with CsA and Ang II for 12 hours and AOPP assay was performed at zero and one hour interval.

**Results:** There was a statistically significant increase in AOPP production in both the CsA and Ang II when compared to the control group with a p value < 0.05.

**Conclusion:** CsA can induce oxidative stress and preventing/controlling it may be necessary to prevent untoward effect of the drug.

**Keywords:** Oxidative stress, Reactive oxygen species, Renin angiotensin system

## INTRODUCTION

Reactive oxygen species play a major role in local inflammation and systemic diseases. Oxygen in the form of ions or peroxides form chemically reactive molecules called Reactive Oxygen Species (ROS). These are produced in the course of normal metabolism which can increase in pathological conditions. These species can bind to lipids, carbohydrates and proteins, damaging them and resulting in advanced oxidative products formation. Though many forms of protein damage are evident in in vitro study, in 1996, by Witko-Sarsat V [1] isolated dityrosine containing protein cross linking products in plasma which was designated as AOPP demonstrating a direct link to renal oxidative stress. Currently, AOPP has been identified as a major uremic toxin involved in renal diseases [2], diabetes mellitus II [3], atherosclerosis [4] and other systemic diseases. Recent studies exposed the role of AOPP in Endoplasmic Reticulum (ER) stress [5], fibrosis and other pathogenesis.

CsA, an immunosuppressant, is usually administered for renal and other organ transplants and for many dermatological conditions. This life saving drug induces a major side effect, renal fibrosis [6], which could be life threatening. Also, they can induce hepatic [7] and cardiac fibrosis [8]. The mechanism behind its induction of fibrosis is varied, but mostly through RAS, especially Ang II and its receptor Angiotensin Type-1 (AT1) [9]. Similar to the renal system, individuals on CsA develop gingival overgrowth known as CIGO [10]. The overgrowth though fibrotic, also have vascular features unlike overgrowth due to other drugs like phenytoin and nifedipine.

Gingiva is constantly exposed to external environment; hence there is a great chance of acquiring superadded inflammation. Unlike the gingiva, kidneys are not exposed to external environment and yet they still develop fibrosis owing to the abundance of ROS, especially NADPH and AOPP suggesting drug induced oxidative stress [11]. The former has been implicated in the pathogenesis of fibrosis while the latter has been linked to ER stress and fibrosis. Both CsA and

Ang II have been proposed to increase ROS production as they increase the intracellular calcium [12].

Gingival inflammation (gingivitis) is predominantly induced by biofilm and if untreated leads to an irreversible situation called periodontitis [13]. Both the conditions are infested with pro inflammatory cytokines, mediators and importantly ROS. Periodontitis not only compromises the teeth, but also been proposed as risk factor for atherosclerosis, diabetes mellitus, pre-term low birth weight babies, renal diseases etc., [14]. Being a chronic condition and with the amount of surface area, the load of bacteria and inflammatory components generated by this oral tissue will be large and sustained which makes it a strong risk factor for systemic diseases.

In spite of numerous reports on ROS in gingivitis/periodontitis, the concept of AOPP is new to periodontium. Considering the effect of CsA and Ang II on renal ROS, especially AOPP, we were keen to observe if, without inflammation, the drug and hormone have a stimulatory effect on human gingival fibroblasts AOPP production.

## MATERIALS AND METHODS

This ex vivo study was carried out in Department of Periodontics, Sri Ramachandra University, Chennai, Tamil Nadu, India, over a period of six months.

### Ethical Approval

This study was approved by the institutional ethics committee of Sri Ramachandra University. After proper informed consent, six patients were selected from the outpatient area of the Department of Periodontology.

### Culture of Human Gingival Fibroblasts

Gingival tissue biopsy samples were obtained from six healthy volunteers undergoing crown lengthening procedure. The volunteers were selected based on the following inclusion criteria: clinically healthy gingiva of patients within the age group of 18 to 35 years

and exclusion criteria: clinical signs or radiographic evidence of gingival/periodontal inflammation, systemic diseases, smoker and on antibiotics or anti-inflammatory for the past six months.

Tissue sample was collected under xylocaine local anesthesia with No: 15 BP blade. The collected samples were preserved in a transport media which contained 1X Phosphate Buffered Solution (PBS) and antibiotics (penicillin-100 IU/ml, streptomycin-100 µg/ml, and amphotericin B-100 µg/ml, gentamycin-200ug/ml, ciprofloxacin-200ug/ml) until they were processed.

Human gingival fibroblast cells were cultured using enzymatic digestion method. Monolayer cultures were incubated in complete growth essential medium at 37°C in an incubator containing humidified air (5% CO<sub>2</sub>). The cultures were observed every day and medium was replenished every two days. Once the Human Gingival Fibroblast (HGF) attained 90% confluence in culture plates they were trypsinised and sub-cultured in T-25 cm<sup>2</sup> flask.

### 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyl Tetrazolium Bromide (MTT) Assay

The MTT cell proliferation assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. MTT assay was performed using the protocol given by Mossman T et al., [15]. HGF cells were seeded in 96 well plates. Then HGF cells were treated with 50 µm, 25 µm, 10 µm, 5 µm and 1 µm of CsA and 100 µm, 10 µm, 1 µm and 100 nm of Ang II for 24 hours. Each of these concentrations was in triplicates. Control, diluent control and blanks wells were included. 20 µl of MTT reagent (final concentration of 0.5 mg/ml) was added to each of the wells. Then the plate was incubated at 37°C for four hours. Almost, 100 µl of Dimethyl Sulphoxide (DMSO) was added to all the wells following which the cells were re-incubated at 37°C for four hours. The plate was placed on a plate mixer for few minutes. The reading was taken at 590 nm in a Multimode plate reader (Perkin Elmer). The average values were obtained from triplicate readings. MTT readings were taken after 24 hours. Percentage of residual cell viability was determined as

[1 - (Optical Density of treated cells/Optical Density of control cells)] × 100. MTT assay revealed inhibitory concentration of CsA and Ang II were 25 µm and 10 µm respectively.

### Sample Preparation for Advanced Oxidative Protein Products

Gingival fibroblasts were cultured in 96 well plates and cells were treated with CsA (25 µm) and Ang II (10 µm) and incubated for 12 hours following which AOPP levels were estimated. All the measurements were done in duplicates (two wells for each group).

### Advanced Oxidative Protein Products Assay

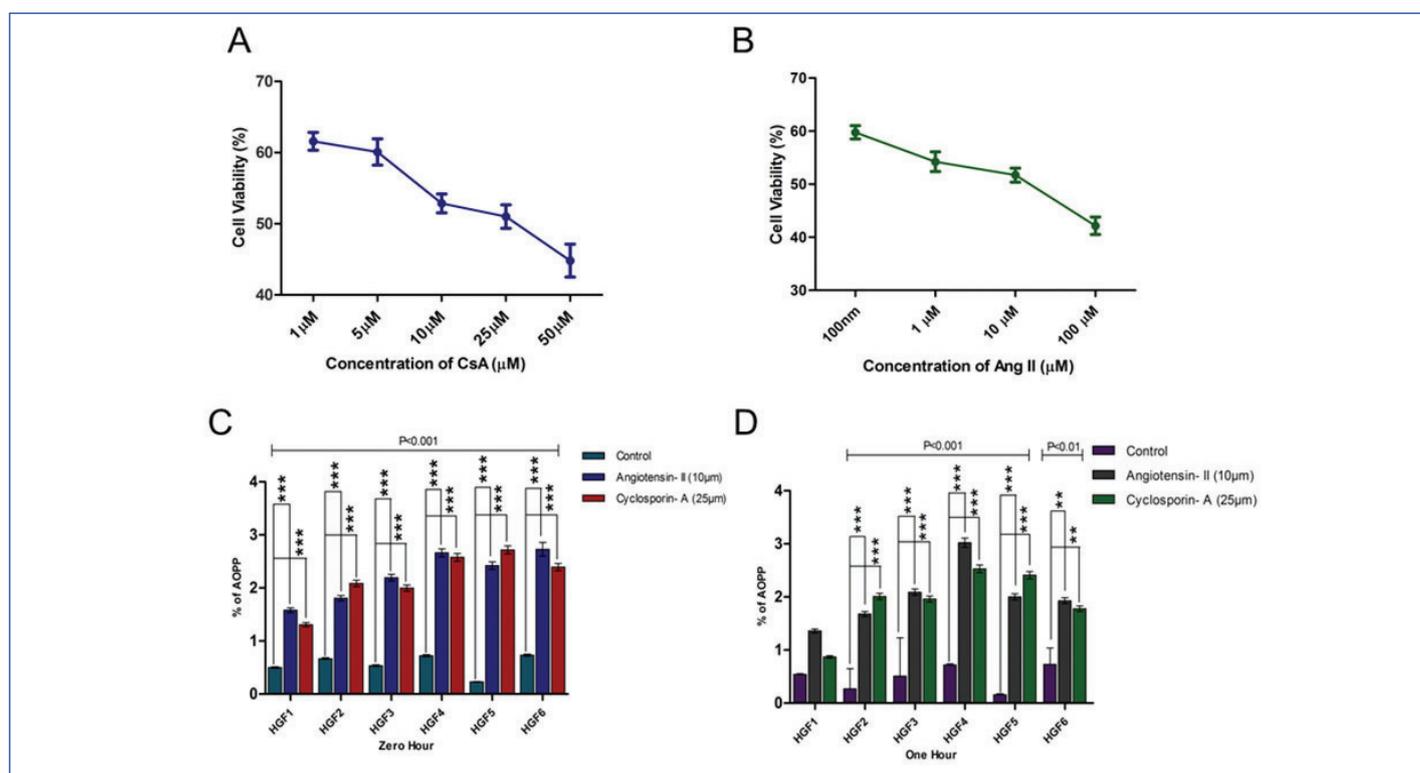
Advanced Oxidative Protein Products (AOPP) was estimated using the assay put forth by Witko-Sarsat V et al., [1]. Spectrophotometry was used to estimate the production of AOPP. The assay was calibrated using chloramine-T and absorbance read at 340nm on a microplate reader at an interval of zero to one hour. AOPP concentrations were expressed as µmol/l of chloramine-T equivalents.

### STATISTICAL ANALYSIS

The statistical significance was evaluated using Two-way ANOVA followed by Bonferroni post hoc tests. Two way ANOVA was also used to evaluate the statistical significance between zero and one hour. Software graph pad prism was used for statistical analysis. All values were expressed as mean±Standard Deviation (SD). The p value was <0.05, considered statistically significant.

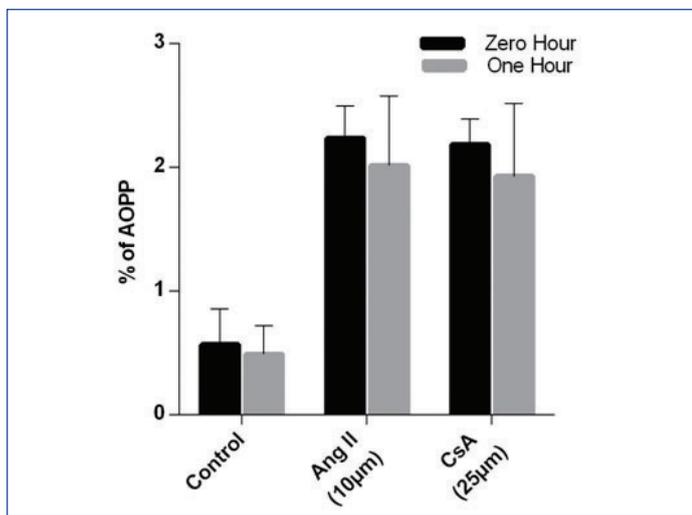
### RESULTS

In the present study different concentration of Ang II and CsA activities against human gingival fibroblast were screened. The percentage of inhibitory properties were found to be concentration dependent, based on the Inhibitory Concentration (IC) 50 values we chose 10 µm and 25 µm concentration [Table/Fig-1a,b]. Cyclosporine and Ang II were added (zero hour and one hour incubation). One group of cells was maintained without any addition of drug or hormone which served as control group. At the end of stipulated time, culture



**[Table/Fig-1]:** Effects of Ang II and CsA on human gingival fibroblast: a,b) AOPP measurements in cultured gingival fibroblast. Gingival cells at 5x10<sup>3</sup> were treated with 25 µm CsA and incubated for 12 hour at 37°C and AOPP levels were measured in both treated and control samples with indicated time levels at zero and one hour. Histogram indicated the expression level of AOPP at 10 µm of Ang II treatment at zero and one hour; c,d) Data are expressed as Mean±SEM for three independent experiments.

\*p>0.05 is considered statistically significant



[Table/Fig-2]: Graphical representation of comparison between zero and one hour.

wells were estimated for AOPP using a microtitre plate reader. There was a statistically significant increase in AOPP levels in both CsA and Ang II groups when compared to the control group ( $p < 0.01$ ) after zero hour and one hour [Table/Fig-1c,d]. The increase in AOPP levels was slightly higher at zero hour after the addition of CsA and Ang II when compared to one hour [Table/Fig-2]. However, the results were not statistically significant.

## DISCUSSION

The present study shows significantly increased production of AOPP in human gingival fibroblasts when stimulated by CsA and Ang II. Though the production of AOPP decreased over one hour period by control cells, both cyclosporine and Ang II increased it. To our knowledge this is the first report of AOPP production by gingival cells. In recent times, AOPP has emerged as one of the most crucial reactive oxygen species. Oxidative modified proteins, AOPPs, are long-term indicators of oxidative stress, which possess pro-oxidant activity, block vasodilatation induced by Nitric Oxide (NO), induce lipid peroxidation, increase pro-inflammatory and adhesive molecules as well as cytokines [1]. Studies on the influence of cyclosporine on ROS generation are varied. CsA's protective effect on oxidative stress could be inferred from the report of Imberti et al., [16], who observed cyclosporine preventing rat hepatocytes from lethal effect of oxidative stress. Contradicting this, Richter R et al., [17] reported CsA attenuating mitochondrial ROS preventing apoptosis/necrosis. Akool el-S et al., [18] documented that CsA induced ROS activate TGF beta gene in mesangial cells.

Animal cell and human cell culture studies have confirmed the influence, cyclosporine exhibit over Ang II generation. Navar LG et al., [19] demonstrated that there was a rise in both the circulating (plasma) and intra-renal (kidney tissue) Ang II levels in CsA-induced hypertensive rats which could be attributed to concomitant increase in renin activity. Likewise, we had demonstrated an increase in Ang II and AT1 receptors in gingiva (communicated). However, unlike Navar LG et al., we showed an increase in Angiotensin Converting Enzyme (ACE) synthesis by human gingival fibroblast. Substantiation for our findings come from a study done by Santos CF et al., [20], that detected most of the components of RAS in rat gingiva.

Navar LG et al., [19] demonstrated that CsA induced hypertension is associated with elevated Ang II and ROS formation in rats and AT1 receptor blockade prevented this increase in ROS levels and development of CsA induced hypertension, suggesting that at least part of increased ROS generation is a consequence of the elevated Ang II levels and it's not mandatory that Ang II to be involved in CsA induced ROS generation which can be substantiated in vitro by CsA stimulated increase in ROS levels in a human mesangial cells [21] and vascular endothelial cells [22]. Results of the present

study show a significant increase in AOPP generation following CsA and Ang II individually, proving that both have their own capacity to generate ROS in human gingival fibroblasts. Literature review shows that increase in intracellular calcium results in increased ROS generation [23]. In our previous study we have demonstrated increased intracellular calcium following CsA treatment in vitro [24]. The influence of CsA on calcium stores seems to vary from cell to cell.

Apart from AT1 receptor blocker, CsA-induced superoxide formation in rat aorta can be blocked by an endothelin receptor antagonist, suggesting a role of endothelin in CsA-induced ROS production [25]. It is well known that Ang II stimulates endothelin production [26]. Earlier we have demonstrated increased endothelin-1 in CsA induced human gingival overgrowth [27] suggesting more than one pathway by which cyclosporine can augment ROS generation in a tissue.

Evidence of AOPPs presence in the plasma of patients with early-stage chronic kidney disease exists [28]. AOPP was found to be a more sensitive indicator of oxidative stress than ox-Low Density Lipoprotein after kidney transplantation [29]. Moreover, AOPP elevation has been reported as an independent risk factor for coronary artery disease in non-uremic patients [4]. Hence, it's significant that the present study also demonstrates that CsA/Ang II induces AOPP production in a tissue that is highly prone for inflammation.

## LIMITATION

The limitation of this study is that an antagonist to Ang II was not employed which would have reiterated the effect of CsA and Ang II on AOPP production. Future studies utilizing nutrients like melatonin as anti-oxidants may have important clinical implications such as restricting gingival overgrowth and controlling the risk factor for many systemic diseases by reducing the serum load of AOPP.

## CONCLUSION

CsA and Ang II induce oxidative stress by the generation of AOPP in human gingival fibroblasts and controlling the oxidative stress may be a good option for preventing CsA induced gingival overgrowth.

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#### PARTICULARS OF CONTRIBUTORS:

1. Head of Department, Department of Periodontics, Sri Ramachandra University, Chennai, Tamil Nadu, India.
2. Research Scholar, Centre for Regenerative Medicine and Stem Cell Research, Central Research Facility, Sri Ramachandra University, Chennai, Tamil Nadu, India.
3. Lecturer, Department of Periodontics, Sri Ramachandra University Chennai, Tamil Nadu, India.
4. Research Scholar, Centre for Indian Systems of Medicine Quality Assurance and Standardization, Sri Ramachandra University, Chennai, Tamil Nadu, India.

#### NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:

Dr. Suresh Ranga Rao,  
Head of Department, Department of Periodontics, Sri Ramachandra University, Chennai-600116, Tamil Nadu, India.  
E-mail: chennaidentist@gmail.com

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