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EVALUATION OF TRANSFORMING GROWTH FACTOR-BETA 1 (TGF-β1) LEVELS IN GCF AND SALIVA DURING INITIAL ORTHODONTIC TOOTH MOVEMENT – *AN IN VIVO STUDY*

Dr. Femin Pushpan Karyat*, Dr. T. Shobbana Devi, Dr. Valai Kasim Shakeel Ahmed, Dr. N. R. Krishnaswamy

India.

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***Corresponding Author**
Dr. Femin Pushpan Karyat
India.
dr.shobathalur@gmail.com,

ABSTRACT

Aim: To determine the changes in concentration levels of Transforming Growth Factor-β1 (TGF-β1) as a reaction to light continuous orthodontic forces during initial stage of alignment in Gingival Crevicular Fluid (GCF) and saliva and also to compare the expression levels of TGF – β1 in GCF and saliva. **Materials and Methods:** GCF and saliva samples were collected from ten healthy subjects (6 females and 4 males, aged 13-23yrs). The samples were collected at the following stages:- **T_0** – Pre Treatment, **T_1** – 7 days after initiation of orthodontic treatment, **T_2** - 30 days after initiation of orthodontic treatment, disposable micropipettes were used for GCF collection and the saliva samples were collected in sterile containers. The collected samples were stored in sterile Eppendorf tubes at -80°C until subjected to ELISA test to determine the concentrations of TGF – β1. **Results:** The mean concentration levels of TGF-β1 in GCF was 25.8 $\pm$ 15.6 pg/ml at T_0, 32.5 $\pm$ 16.1 pg/ml at T_1 and 22.3 $\pm$ 11.4 pg/ml at T_2. The mean salivary concentration levels of TGF-β1 was 22.2 $\pm$ 12.2 pg/ml at T_0, 30.1 $\pm$ 15.8pg/ml at T_1 and 35 $\pm$ 13.2 pg/ml at T_2. The comparison between GCF and salivary levels of TGF-β1 (Intergroup) did not show any statistically significant difference. **Conclusion:** The light continuous forces applied during initial stage of alignment brought about changes in the levels of TGF-β1 expression in both GCF and Saliva. There is a positive indication that saliva could be used as a viable substitute for GCF when used for assessing bone remodeling in patients undergoing orthodontic treatment.

KEYWORDS: Orthodontic tooth movement; Gingival crevicular fluid; Saliva; TGF-β1.

INTRODUCTION

Orthodontic force application triggers a cascade of events by mechanical deformation of cells and extracellular matrix, leading to release of the first messengers (Prostaglandins and Leukotriene's) followed by the second messengers (cyclic AMP, Inositol Phosphate, Diacylglycerol and Mitogen-activated tyrosine kinases). Orthodontic force invokes an inflammatory response associated with the production and release of a variety of cytokines. Cytokines are cell signaling molecules that aid cell to cell communication in immune response and stimulate the movement of cells towards the site of inflammation.

Cytokines are small soluble proteins produced by broad range of cells. Including macrophage, B and T lymphocytes, endothelial cells etc. The cytokine molecule group are Interleukin's, Interferon's, Growth factors, Cytotoxic factors, Activating or Inhibitory factors, Colony stimulating factors and Intercrines.[1] Transforming growth factor-β (TGF-β) is a pleiotropic growth factor and belongs to a family of dimeric 25 kDa polypeptides. In mammalian cells, there are three subtypes of TGF-β ligands, β1, β2 and β3.[2] The three major activities of TGF-beta are 1. Modulating the proliferation and/or migration of structural cells in the periodontium; 2. Exert immunosuppressive effects and 3. Enhance the formation of extracellular matrix.[3] TGF-β is by far the most abundant cytokine in bone, by its mere abundance (200 mg/kg), it can be considered as a key factor in bone turnover.[4] TGF-β1 is synthesized as a large precursor molecule, which is cleaved into active TGF-β1 and latency-associated protein (LAP).[5]

In the recent past, In-vivo studies have used Gingival Crevicular fluid (GCF) to detect molecules involved in both bone modeling and remodeling process. GCF is an osmotically mediated inflammatory exudate present in the gingival sulcus with a flow rate of 0.13 ± 0.37 µL/min in healthy gingival tissue.[6] Orthodontic force induce the movement of periodontal ligament (PDL) fluids coronally or apically and along with them any cellular biochemical product produced from mechanical perturbation. Although GCF might be a potential medium for assessing inflammatory markers, but there are certain inherent limiting factors associated with it.

Saliva contains constituents of exocrine glands in the oral cavity, GCF and some inflammatory markers. Saliva is readily available and easily collected without specialized equipment. Clinical studies have shown that TGF–β1 can be detected in GCF during orthodontic tooth movement. Literature evidence is limited to studies which evaluated level of TGF–β1

expression in saliva during initial orthodontic tooth movement. This study was designed.

1. To evaluate the changes in concentration levels of TGF-β1 in GCF and Saliva as a reaction to light continuous forces applied during initial stage of orthodontic tooth movement (OTM).
2. To compare the expression levels of TGF–β1 in GCF and saliva.

MATERIALS AND METHODS

In the present study 10 healthy subjects (6 females and 4 males) in the age group of 13-23yrs were enrolled. The study protocol was approved by the Institutional Review Board (IRB) of Ragas Dental College and Hospital, Chennai and informed consent was obtained from all the subjects. Subjects with arch length tooth size discrepancy which warranted extraction of all first bicuspids to correct the same were included. Subjects were in good health with no untoward periodontal disease and probing depths were < 3mm with no previous history of orthodontic treatment. Subjects were excluded if they had taken any anti-inflammatory and antibiotic medication during the month preceding the start of the study and if they had active signs of periodontal disease.

Study Design

All the subjects were treated using fixed orthodontic appliance – Ovation series (0.022" x 0.028" Roth Prescription) fully programmed brackets. An 0.014" NiTi archwire (Orthonol NiTi Preformed natural arch form, RMO) was used initially in the aligning stage in both upper and lower arch for all the patients. All subjects received repeated oral hygiene instructions, which included the correct use of a toothbrush and an interdental brush. The subjects were instructed not to eat or drink for 1 hour before the sample collection. GCF and saliva were collected at the following time points.T_0 – Pre Treatment, T_1 – 7 days after initiation of orthodontic treatment and T_2 - 30 days after initiation of orthodontic treatment.

Sample Collection

GCF sampling

GCF samples were obtained from lower anterior teeth following the isolation and drying of the site. GCF were collected using disposable micropipettes (Ringcaps® - Hirschmann laborgerate, Eberstadt Germany). The micropipettes were inserted into the entrance of the gingival sulcus without any resistance, 4-5µL of GCF were collected from the 6 lower anterior teeth (Fig 1). The collected GCF was pipetted into 200 µl of elution buffer (50ml Hcl) and stored in sterile Eppendorf tubes at -80°C. The patients were instructed to brush their teeth and

not to eat anything 1 hour before the sample collection. The same protocol was followed for collecting the GCF at different time points.

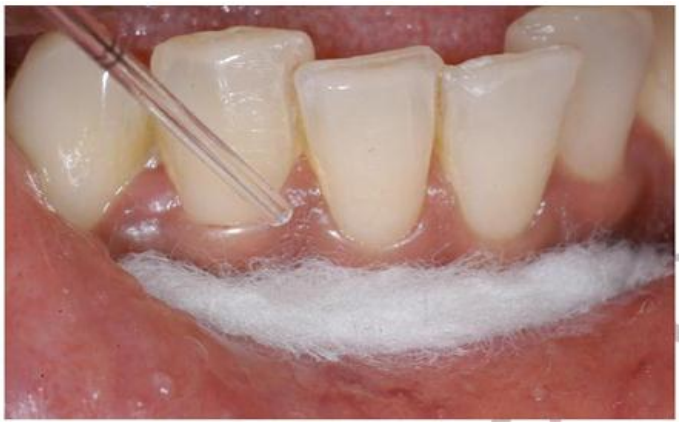

Fig 1: GCF Collection Procedure Using Micropipettes.

Saliva collection

Saliva was collected by asking the patient to rinse twice with water and to drink 2 to 3 ounces of water to avoid contamination with respiratory secretions. Patients were instructed to allow 5 ml of saliva to flow into a sterile cup without actively expectorating. The saliva samples were transferred to the vacutainers which were then centrifuged for 3-5 minutes at 1300 rpm, and the supernatants were collected and transferred to Eppendorf tubes and stored at -80°c until analysis.

Biochemical Analysis

ELISA (Enzyme Linked Immuno Sorbent assay) test for TGF-ß1

The stored GCF and saliva samples were thawed at room temperature and then assayed for TGF-β1 using the Human TGF-β1 ELISA kit (Genxbio health sciences pvt ltd, Cusabio).

Statistical Analysis

Statistical analyses were performed using the SPSS software version 19. For each variable measured from GCF and Saliva samples taken at various time points (T_0, T_1 and T_2) the mean and standard deviation were calculated as shown in Table 1.

One way Anova was done for overall comparison of the GCF and saliva samples at different time points. Tukey HSD post hoc test were used for multiple comparisons between the means of different time points within the groups. Independent t test were done to compare the levels of TGF-β1 between GCF and Saliva at each time point. Level of statistical significance was set at $P < 0.05$.

RESULTS

Table 1: Mean concentration levels of TGF-β1 in saliva and GCF at various time points.

		Group			
Observation Periods		Saliva (in pg/ml)		GCF (in pg/ml)	
		Mean	SD	Mean	SD
	T_0	22.2	12.2	25.8	15.6
	T_1	30.1	15.8	32.5	16.1
	T2	35	13.2	22.3	11.4

Statistical significance was set at $P < 0.05$.

Concentration levels of TGF-β1 at various time points in GCF

The mean concentration levels of TGF-β1 was 25.8 ± 15.6 pg/ml at T_0, 32.5 ± 16.1 pg/ml at T_1 and 22.3 ± 11.4 pg/ml at T_2.(Table 1 & Fig. 2). The mean concentration level of TGF-β1 had increased at T_1 when compared to T_0 and then the values declined by T_2. The comparison of the mean concentration levels of TGF-β1 at the different time points within the group did not show any statistical significance.

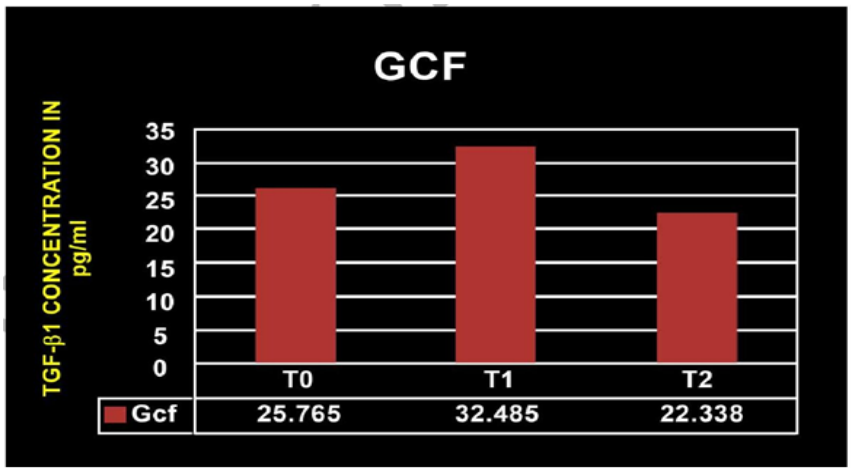

Figure 2: TGF-β1 concentration levels in GCF at different time points.

Concentration levels of TGF-β1 at various observation periods in saliva

The mean concentration levels of TGF-β1 was 22.2 ± 12.2 pg/ml at T_0, 30.1 ± 15.8pg/ml at T_1 and 35 ± 13.2 pg/ml at T_2. (Table 1 & Fig. 3) The mean concentration levels of TGF-β1 had increased at T_1 when compared to T_0 and the values showed further increase at T_2. The comparison of the mean concentration levels of TGF-β1 at different time points within the group did not show any statistical significance.

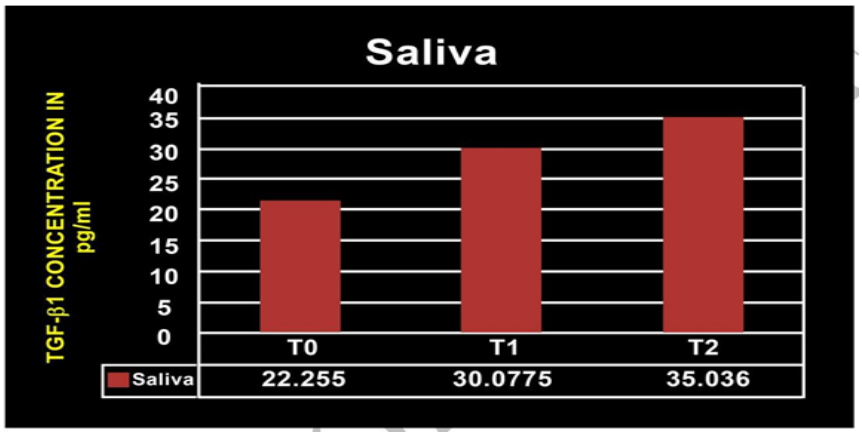

Figure 3: TGF-β1 concentration levels in saliva at different time points.

Comparison between GCF and saliva groups

The TGF–β1 levels showed an increase at T1 (day 7 after placing initial NiTi archwire) in both GCF & Saliva when compared to baseline levels and it gradually decreased at T2 (day 30) in GCF, where as in Saliva the levels further increased.(Fig. 4) No statistically significant difference were found between the two groups at all the three observation periods (P > .05).

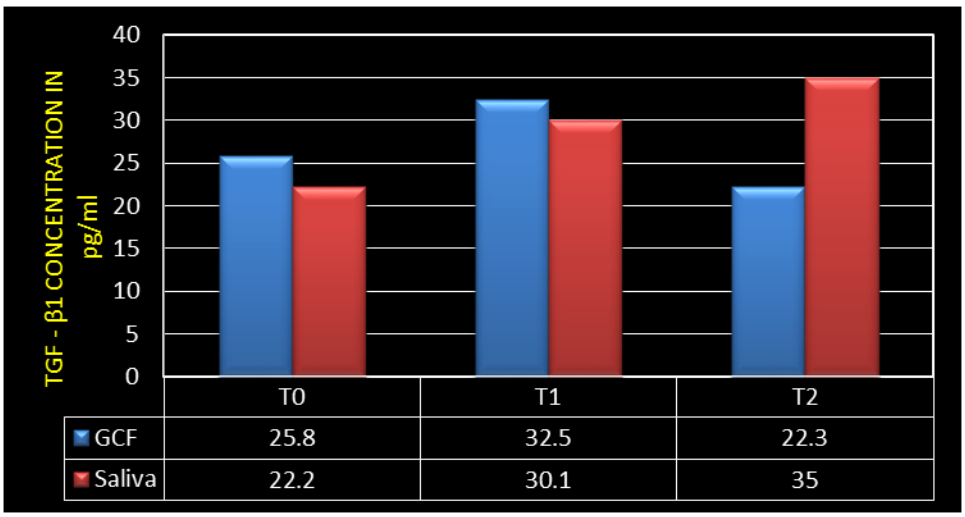

Figure 4: Comparison of TGF-β1 concentration levels in GCF and saliva at different time points.

DISCUSSION

Tooth movement induced by orthodontic force is characterized by remodeling process within the periodontium.[7] The inflammatory response following force application during OTM is a pre-requisite for bone remodeling. Vascular and cellular changes are accompanied by the emancipation of inflammatory mediators like the cytokine like growth factors and neuropeptides in the periodontium.[8] Mechanical forces are transduced from the strained

extracellular matrix (ECM) to the cytoskeleton through cell surface proteins. The ECM is primarily a collection of fibrous proteins embedded in a hydrated polysaccharide gel. Interestingly, bone matrix also contains several growth factors – including TGF-β, bone morphogenic protein, fibroblast growth factors. These molecules are stored in the matrix to be used during the remodeling process. The cellular events following the OTM are controlled by the basic multicellular units (osteoclast, osteoblast, osteocyte) and its activity can be measured biochemically by determining biomarkers of bone remodeling.

In this study TGF-β1 was selected as the inflammatory marker (biomarker) to be assessed, as this cytokine has been shown to be closely associated with the remodeling and expression of ECM of mesenchymal tissue and alveolar bone. Amongst the various mechanically stress induced cytokines in the PDL, IL-6 and TGF-β1 were closely associated with early phases of remodeling.[10] TGF-β1 regulates a broad range of biological processes, including cell proliferation, cell survival, cell migration, cell differentiation and production of ECM. TGF-β1 is released and activated when bone matrix is subsequently resorbed by the osteoclasts which were activated initially by other mediators like prostaglandins and other cytokines. A unique activation mechanism has been proposed in bone, in which resorbing osteoclasts may activate TGF-β1 in their acidic micro environment. TGF-β1 is synthesized as a large precursor molecule and acidification might break the non-covalent bonds between LAP (latency associated protein) and mature TGF-β1, thus releasing the active peptide.[11]

The gingival sulcus was selected as the site in this study because of its continuity with the PDL and its accessibility within the oral cavity. Cytokine values found in the sulcus provide an indirect measurement of changes in the PDL as a result of bone resorption. However the rationale for using GCF on a routine basis in clinical situations is still far-fetched due to various difficulties. Few of the disadvantages include, small volume of fluid only available. It is also difficult to collect an adequate volume of GCF in a short period, unless the sites are inflamed and contain large volumes of GCF and patient compliance is required. GCF collection becomes difficult with the orthodontic appliances in place. Extreme care is required for an atraumatic collection of GCF, any injury to the site can lead to contamination of the GCF by serum. Therefore the objective of this study was to find an alternative medium to determine these biological changes, so in this study TGF-β1 levels in saliva was evaluated to know whether it can be used as an appropriate alternative. The different mediators involved in alveolar bone remodeling are continuously washed into the saliva from the GCF, as concluded

by Ruhl et al[12]. In their study the inflammatory cytokines detected in saliva did not come from the secretions of major salivary glands and proposed that GCF was the likely source of these cytokines.[12]

In this study, samples were collected at three different time points which coincides with the different phases of tooth movement. The mean concentration levels of TGF-β1 was 25.8 ± 15.6 pg/ml at T_0, 32.5 ± 16.1 pg/ml at T_1 and 22.3 ± 11.4 pg/ml at T_2 (Table 1). In healthy periodontal tissue the source of TGF-β1 is the mesenchymal stromal cells. TGF-β1 along with other cytokines is considered to play crucial roles in the maintenance of tissue homeostasis. The mean concentration level of TGF-β1 had increased at T_1 when compared to T_0. The reason for this increase in TGF-β1 expression is due to the inflammatory changes associated with OTM. After the onset of lag phase, only removal of necrotic tissue and bone resorption from adjacent marrow spaces allow the resumption of tooth movement. Initially the mechanically stressed region gets infiltrated by the neutrophil, which later on gets replaced by the macrophages. These macrophages are the primary source for the increased TGF-β1 levels by day 7. The findings in this study at T_0 and T_1 are in agreement with the results shown by German Barbieri et al.[13] In their study, they have evaluated TGF- β1 levels in GCF after placing elastic separators and shown increase in the levels by day 7.

Later on, the latent form of TGF-β1 from the bone gets activated and released facilitating further tooth movement.

As the force levels decay by the end of one month, the infiltration of inflammatory cells into the region also reduces. This in turn reduces bone resorption by the osteoclastic cells. Unless the bone matrix is resorbed, no more TGF-β1 is released. So by the end of one month (before the next appointment) the TGF-β1 levels come down to the baseline levels. In this study the mean GCF levels of TGF-β1 declined by T_2 and it was lower than T_1 levels, reflecting the reduction in resorptive and remodeling processes. This might be a relevant clinical finding which indicates the need for re-activation of the orthodontic appliance and as GCF is considered to be a site-specific medium, observing the changes in the biomarker levels from a specific site might suggest whether the applied force is optimal for that particular tooth or a set of teeth.

The Salivary TGF-β1levels assessed in our study showed that the mean concentration levels of TGF-β1 was 22.2 ± 12.2 pg/ml at T_0, 30.1 ± 15.8 pg/ml at T_1 and 35 ± 13.2 pg/ml at T_2. The

mean concentration levels of TGF-β1 had increased at T_1 when compared to T_0 and it reflects the GCF values. The salivary TGF-β1 values showed further increase at T_2 when compared to T_0 and T_1 values which could be due to the pooled sample: As quoted by Krishnan et al[7] when compared to GCF which is site specific (lower anterior region in this study), saliva represents a pooled sample from all periodontal sites inspite of dilution.[5]

A second reason may be, as the orthodontic treatment is commenced, inflammatory changes in the oral epithelia can be observed in a mild form.[6]

The GCF and Salivary TGF-β1 concentration levels at various observation periods obtained by Elisa test, when analyzed statistically within the group (Intra group) did not show any significance, even though there was a marked change in the mean values at different observation periods. This might be due to the problems associated with the 'P' value as suggested by Nikolaos Pandis.[14] Pandis et al, quoted that P values are sensitive to sample size and the standard deviation. Although 'P' value might indicate a statistically significant result it provides no insight into the clinical relevance. Therefore, even small differences of no clinical importance can appear important if there is a large enough sample size.

In this study, the mean TGF-β1 levels increase in GCF and Saliva by day 7 and also there is a positive indication that saliva can be used as a viable substitute for GCF when used for assessing bone remodeling for induviduals undergoing orthodontic treatment. Assessing the local response of bone to orthodontic force by evaluating the levels of TGF-β1 helps determine the long term effects of different force delivery systems on OTM.

CONCLUSION

Based on the findings of this study it can be concluded that TGF-β1 is an integral component in the remodeling process, as the concentration levels of this biomarker changes in concordance with the different phases of orthodontic tooth movement and also the changes in the level of TGF-β1 are reflected in both the GCF and Saliva. There is a positive indication that saliva can be used as a viable substitute for GCF in assessing the bone remodeling process associated with OTM.

However, further longitudinal studies with larger sample sizes are required to know about the full potential of saliva, to isolate saliva from pooled samples for assessment of biomarkers associated with progressive OTM.

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