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Effect of Non-Surgical Periodontal Treatment on Salivary Hypoxia Inducible Factor (HIF-1α) in Patients with Generalized Chronic Gingivitis and Periodontitis

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Abstract

Aim: *The study aimed to estimate and compare the levels of salivary hypoxia inducible factor-1 alpha (HIF-1α) in unstimulated saliva samples of periodontally & systemically healthy volunteers, generalized chronic gingivitis & periodontitis patients before and after non-surgical periodontal treatment (NSPT).* ***Materials and Methods****: A total of 24 subjects were categorized into 3 groups: Group I: 8 periodontally and systemically healthy volunteers; Group II: 8 generalized chronic gingivitis patients and Group III: 8 stage III grade B periodontitis patients. The periodontal parameters were recorded at 0 day in all three groups and reassessed following non-surgical periodontal treatment on 90th day in group II and group III. The saliva samples were collected on 0 day in all the three groups and on 30th day & 90th day in group II and group III. Molecular analysis was done for detection of HIF-1α using enzyme linked immunosorbent assay (ELISA).* ***Results****: On comparing the periodontal parameters at 0 day, the mean periodontal parameters were higher in both group II & III. Following the NSPT, both group II and III showed improved periodontal parameters with significant reduction at 90th day. Intra and intergroup comparison of the level of salivary HIF-1α was found be to be statistically significant in both group II & III compared to group I at 0, 30th and 90th days.* ***Conclusion****: The present study indicates that HIF- 1α can be used as potent biomarker to assess the effect of periodontal treatment and also signifies its possible interlink between hypoxia and periodontal disease pathogenesis.*

Keywords*: Non-Surgical Periodontal Treatment, Salivary Hypoxia Inducible Factor, Generalized Chronic Gingivitis, Periodontitis.*

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INTRODUCTION

Periodontitis is a multifactorial disease that includes bacterial etiology, host immune response and environmental factors.[1] It is characterized by a chronic inflammatory condition that involves the gingiva and the tooth-supporting apparatus. It is initiated by localized gingival inflammation which could be due to bacterial invasion from dental plaque and biofilm present on the tooth structure. Inflammation can be attributed to causative agents such as injuries, toxins from microorganisms or any systemic diseases which may result in creating a hypoxic environment.[1] Hypoxia and inflammation frequently coexist in chronic inflammatory diseases and modulate each other.

Hypoxia-Inducible Factor (HIF) is a heterodimeric complex, composed of oxygen-destructible α-subunit and an oxygen-indestructible β-subunit. There are three isoforms of the α-subunit and two isoforms of the β-subunit. HIF acts as a principal regulator of cellular and systemic responses such as hypoxic adaptation, regulating gene expressions involved in glycolysis, erythropoiesis, angiogenesis, cell proliferation & function, apoptosis and cell survival during lower oxygen environment.[2]

When the hypoxic environment in periodontitis persists chronically, it intensifies the anaerobic pathogen's survival and further lowers the oxygen tension in the vicinity. Under hypoxic conditions, human periodontal ligament cells express immuno-modulatory signals and stimulate pro-inflammatory gene expressions such as interleukin- 1β, interleukin-6, prostaglandins E2, Receptor Activator of Nuclear factor- Kappa B (RANK) and osteoprotegrin (OPG) resulting in periodontal breakdown. It also increases the expression of angiogenic factors to improve the blood supply in the inflamed periodontal tissues. These include Vascular Endothelial Growth Factor (VEGF), Platelet Derived Growth Factor (PDGF), angioprotein 1 & angioprotein 2 which are responsible for angiogenesis and wound healing.

During tissue/cellular hypoxia there will be activation of hypoxia-inducible factors and an upsurge in the expression of HIF- 1α, which is involved in periodontal inflammation. It has a dual role of mediating the host's immune response against microbial invaders for maintaining periodontal health and also facilitates periodontal tissue breakdown, thus resulting in the progression of periodontitis.[2] Hence, this study aims to determine the expression of salivary HIF-1α as an inflammatory biomarker in unstimulated saliva of periodontally and systemically healthy volunteers, generalized chronic gingivitis and periodontitis patients.

2. MATERIALS AND METHODS

A total of 24 male and female subjects aged between 20-65 years were selected for the study from the Department of Periodontology, Meenakshi Ammal Dental College, Chennai based on the inclusion & exclusion criteria.

The study was approved by the "Institutional Ethical Committee of Meenakshi Ammal Dental College & Hospital, Chennai (MADC/IEC-I/022/2021). The study procedure was explained in detail to all the participants and a written informed consent was obtained from each subject before enrolment if they were willing to participate in the study.

The study subjects were divided into:

- Group I: 8 Periodontally and systemically healthy volunteers,
- Group II: 8 Generalized chronic gingivitis patients and
- Group III: 8 Stage III Grade B periodontitis patients.

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The inclusion criteria was as follows: Individuals willing to participate in the study, subjects within the age group of 20-65 years, subjects having ≥ 10 remaining natural teeth. Additionally, the selection of periodontitis groups was based on the 2017 classification of periodontal and peri-implant diseases. [3]

For Group I, periodontally healthy subjects with no clinical attachment loss (CAL=0), probing depth (PD) ≤ 3 mm, bleeding on probing (BOP) at ≤10% of tooth site with no radiographic bone loss, for Group II, generalized chronic gingivitis patients with no attachment loss, probing depth ≤ 3mm, bleeding on probing ≥30%, and with no radiographic bone loss and for group III, patients with stage III grade B periodontitis with generalized interdental CAL ≥5mm with radiographic bone loss extending to middle third of root and beyond, tooth loss due to periodontitis present ≤ 4 teeth, probing pocket ≥ 6mm, vertical bone loss ≥3mm with class II/III furcation and moderate ridge defect with clinical attachment loss < 2mm over 5 years were selected.

The exclusion criteria for all groups in the study were as follows: Subjects with systemic conditions such as diabetes mellitus, respiratory diseases, renal disease, liver disease, rheumatoid arthritis, allergy, GOUT, advanced malignancies and HIV infection, current smokers and individuals who quit smoking less than 6 months, patients who had undergone periodontal therapy within the previous 6 months, and pregnant women. Periodontal parameters were recorded using a Williams periodontal probe. The periodontal parameters included: (1) Plaque Index (Silness and Loe, 1964)[4]; (2) Gingival index (Lobene et al,1986)[4]; (3) Bleeding on probing (Ainamo and Bay, 1975)[5] and (4) Probing depth (PD) (5) Clinical Attachment Level (CAL). PD and CAL were recorded from six different sites per tooth (mesiofacial, facial, distofacial, mesiolingual, lingual, and distolingual).

Unstimulated 5ml of whole saliva sample was collected from the participants in all three groups two hours after the last meal. The collected saliva samples were then transferred to Eppendorf tubes and centrifuged at 5000 rpm for 10 minutes. The centrifuged samples were stored at -80℃ until further analysis. Periodontally healthy volunteers did not receive periodontal intervention. Eight patients each from Group II and Group III patients underwent scaling and root planing (SRP). Standard oral hygiene instructions were given to both group II & III patients. The saliva sample collection was repeated for Group II and Group III patients on 30th day following the completion of SRP. On 90th day patients were recalled; periodontal parameters were re-recorded and saliva samples were collected from Group II and Group III patients for the estimation of hypoxia inducible factor 1α levels.

Serum and GCF samples were analysed for hypoxia inducible factor (HIF-1α) using enzyme-linked immunosorbent assay (ELISA) kit (Abbkine) according to the manufacturer's instructions. The optical density (OD value) of each well was measured at once using an ELISA plate reader (LABSERV) at 450 nm. Statistical analysis was done using SPSS software (IBM SPSS Statistics for Windows, Version 26.0, Armonk, NY: IBM Corp. Released 2019). The Normality tests Kolmogorov-Smirnov and Shapiro-Wilks tests results revealed that the variables follow Normal distribution. To compare the mean values between groups one way ANOVA was applied followed by Tukey's HSD post hoc tests for multiple pairwise comparisons. To compare mean values between two groups independent samples t-test was applied. Significance level was fixed as 5% ($\alpha = 0.05$).

RESULTS

Intra and Intergroup comparisons of mean difference of all the periodontal parameters including PI, GI, PPD and CAL in Group I, II and III at 0th and 90th day was found to be statistically significant. (Table 1, Table 2 and Table 3)

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Table 1: Mean, Standard deviation & test of significance of mean difference of periodontal parameters at different time points within different groups (Intragroup comparison)

S.no	Variable	Groups	Time Points	Mean±S.D	Mean difference	p-Value
1.	Plaque index	Group I	0 day	0.24±0.09	-	-
		Group II	0 day 90th day	0.72±0.07 0.18±0.04	0.54	<0.001*
		Group III	0 day 90th day	0.95±0.12 0.35±0.14	0.60	<0.001*
2.	Gingival index	Group I	0 day	0.25±0.08	-	-
		Group II	0 day 90th day	0.88±0.07 0.28±0.09	0.60	<0.001*
		Group III	0 day 90th day	1.73±0.20 0.84±0.13	0.88	<0.001*
3.	Bleeding on probing (%)	Group I	0 day	8.31±1.34	-	-
		Group II	0 day 90th day	35.41±2.26 13.61±1.51	21.80	<0.001*
		Group III	0 day 90th day	62.11±9.45 27.86±5.24	34.25	<0.001*
4.	Probing depth (mm)	Group I	0 day	2.36±0.15	-	-
		Group II	0 day 90th day	2.96±0.06 2.31±0.11	0.65	<0.001*
		Group III	0 day 90th day	7.96±0.66 3.69±0.38	4.27	<0.001*
5.	Clinical attachment level (mm)	Group I	0 day	2.36±0.15	-	-
		Group II	0 day 90th day	2.96±0.06 2.31±0.11	0.65	<0.001*
		Group III	0 day 90th day	8.63±0.66 4.85±0.67	3.77	<0.001*

Table 2: Intergroup comparison of periodontal parameters in group I, II & III at 0 day.

S.no	Variables	Pairs		Mean Difference	p-value
1.	Plaque Index	Group I	Group II	-0.48	<0.001*
			Group III	-0.71	<0.001*
		Group II	Group III	-0.23	<0.001*
2.	Gingival Index	Group I	Group II	-0.63	<0.001*
			Group III	-1.48	<0.001*
		Group II	Group III	-0.85	<0.001*
3.	Bleeding on Probing (%)	Group I	Group II	-27.10	<0.001*
			Group III	-53.80	<0.001*
		Group II	Group III	-26.70	<0.001*
4.	Probing Depth (mm)	Group I	Group II	-0.60	0.015*
			Group III	-5.60	<0.001*
		Group II	Group III	-5.00	<0.001*
5.	Clinical Attachment Level (mm)	Group I	Group II	-0.60	0.016*
			Group III	-6.27	<0.001*
		Group II	Group III	-5.67	<0.001*

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Table 3: Intergroup comparison of periodontal parameters in group II & III at 90th day

S.no	Variables	Pair	Mean difference	p-Value
1.	Plaque Index	Group II Group III	0.16	0.012
2.	Gingival Index	Group II Group III	0.57	<0.001*
3.	Bleeding on Probing (%)	Group II Group III	14.25	<0.001*
4.	Probing Depth (mm)	Group II Group III	1.37	<0.001*
5.	Clinical Attachment Level (mm)	Group II Group III	2.54	<0.001*

On intragroup comparison of the mean difference of salivary HIF-1α in Group II and Group III at 0th and 90th days, it was found to be statistically significant (Table 4). Intergroup comparison of mean difference of salivary HIF-1α levels between Group I with Group II and Group III at 0 day and 30th day was found to be statistically significant. However, the mean difference of salivary HIF-1α levels at 90th day between Group II and Group III was found to be statistically insignificant (Table 5).

Table 4: Mean, standard deviation & test of significance of mean difference of salivary HIF-1α at different time points within group II and group III (Intragroup comparison)

S.no	Groups	Time Points	Mean±S.D	Mean difference	p-Value
1.	Group I	0 day	1.89±0.68	-	-
2.	Group II	0 day 30th day	4.13±0.96 1.93±0.78	2.19	0.002*
		0 day 90th day	4.13±0.96 1.51±0.67	2.61	0.001*
		30th day 90th day	1.93±0.78 1.51±0.67	0.42	0.005*
3.	Group III	0 day 30th day	5.65±1.16 3.91±1.03	1.74	0.002*
		0 day 90th day	5.65±1.16 1.76±0.63	3.89	<0.001*
		30day 90th day	3.91±1.03 1.76±0.63	2.15	0.002*

Table 5: Intergroup comparison of salivary HIF-1α in group I, II & III at 0 Day, 30th Day and 90th day

S.no	Time points	Pair		Mean difference	p-Value
1.	0 Day	Group I	Group II	-2.24	<0.001*
			Group III	-3.77	
		Group II	Group III	-1.53	0.011*
2.	30th Day	Group II Group III		1.99	0.001*
3.	90th Day	Group II Group III		0.25	0.47

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DISCUSSION

Periodontitis is one of the ubiquitous disease characterized by persistent destruction of the supporting tissues of the teeth characterized by progressive attachment loss and bone destruction leading to tooth loss.[6,7] Active inflammation is characterized by substantial shift in the tissue metabolism which include changes such as diminished availability of oxygen in the vicinity known as hypoxia.[8,9] Cellular hypoxia occurs frequently and induces significant change, which can be immediate or delayed; that includes cell growth & apoptosis, cell proliferation & survival, pH regulation & energy metabolism, cell migration, matrix & barrier function, angiogenesis, and vasomotor regulation.

Hypoxia activates the hypoxia signaling pathway, which is predominantly governed by hypoxia inducible factor (HIF) stabilization.[10] The hypoxia-inducible factors (HIFs) belong to the transcriptional regulation family whose levels are regulated in response to hypoxic stimuli. On stimulation, it enhances the transcriptional program that allows the cell to respond to the hypoxic environment.[11]

Hypoxia is considered as a pathogenic factor for periodontitis due to the multiplying gram negative anaerobic pathogens in the untreated periodontal pocket causes a significant change in the oxygen level with compromised and increased demand for oxygen supply. This leads to inflammation-associated tissue hypoxia, disturbances in the microcirculation and an increased leukocyte infiltration, particularly the myeloid cells such as polymorphonuclear leukocytes (PMNs) & monocytes.[12] To compensate and sustain the insufficient oxygen environment, hypoxia activates a specific complex known as Hypoxia Inducible Factors (HIF), which plays an integral role in the body's response to low oxygen concentrations, or hypoxia.[13] It was first demonstrated by **Ng KT et al** that HIF-1α was expressed in chronic periodontitis patients and it plays an important intracellular function during periodontal inflammation.[14] A study by **Vasconcelos RC et al** demonstrated that HIF-1α expression was regulated by factors such as hypoxia, bacterial endotoxins and inflammatory cytokines.[15]

Therefore, this study was carried out with an aim to assess and compare the salivary levels of hypoxia inducible factor- 1α (HIF-1α) in generalized chronic gingivitis and periodontitis subjects before and after non-surgical periodontal treatment. Non-surgical periodontal treatment is considered as one of the prime line approach for treating the periodontal disease and it aims at elimination of microbial biofilm and plaque from the subgingival root surface of diseased teeth.[16,17] The mean plaque index values were higher in group II and group III compared to group I and the values decreased from 0^{th} to 90^{th} day following scaling and root planing. This finding was in accordance with the study conducted by **Westfelt E et al 1983** who observed the effect of plaque control in advanced periodontitis and concluded that there was a reduction in plaque accumulation following subgingival instrumentation due to disruption in the formation of plaque and biofilm subgingivally.[18]

The mean gingival index value before non-surgical periodontal therapy was considerably higher in group II and group III compared to group I which could be due to proliferating putative organism and presence of microbial plaque. Following scaling and root planing, the values were found to be reduced in both the groups. This finding was in accordance with the study conducted by **Pihlstorm BL et al 1983** who observed reduced gingival inflammation and decreased gingival index score following periodontal therapy due to decrement of pathogenic organism inducing periodontal inflammation.[19] The presence of bleeding on probing before non-surgical periodontal treatment was due to gingival inflammation, residual subgingival calculus and granulation tissue which irritates the periodontium and punctures the smaller blood vessels. In our study, an average of 50% reduced bleeding on probing was observed following scaling and root planing.

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This finding was in accordance with the study conducted by **Isidor F et al 1984** who observed decreased bleeding on probing during post-operative observation period which ranged from 12-80% after 3months follow-up period; this was due to elimination of subgingival calculus and expulsion of pocket epithelium.[20] The mean probing depth and clinical attachment level in group II and group III was considerably higher compared to group I and their values decreased in both the groups following scaling and root planing. This finding was in accordance with the study by **Proye M et al** who observed reduction in probing depth and gain in clinical attachment level following periodontal therapy.[21]

Another study by **Afacan B et al** observed increased periodontal parameters (plaque index, gingival index, bleeding on probing, probing depth and clinical attachment level) in chronic periodontitis patients and reported reduced values following non-surgical periodontal treatment.[22] Following SRP, the engorged capillaries with inflammatory cell infiltrate in the inflamed gingival connective tissue will be restored by newer collagen rich tissues along with shrinkage of tissues in apical direction. These changes are accompanied by replacement of pocket epithelium to long-junctional epithelium. Formation of long junctional epithelium and bulk collagen fibers in gingival connective tissue improves the resistance of the tissues during the insertion of probe.[22]

Among the oral fluid sources available for the investigation, saliva is considered to have advantage and preferred over gingival crevicular fluid (GCF) & serum due to non-invasiveness, ease of collection and are available in adequate quantities. Hence, in the present study, unstimulated whole saliva was collected from all 24 subjects to estimate the levels of HIF-1α. The salivary HIF-1α levels in group II and group III was higher compared to group I due to proliferating gram-negative anaerobic pathogens followed by inflammatory cell infiltration. This leads to disrupted microcirculatory perfusion which creates raised intake of oxygen, tissue ulceration and lower oxygen tension in the vicinity of the periodontium. Due to this, there will be upsurged accumulation and stabilization of HIF-1α expression.

A study conducted by **Afacan B *et al*** observed an increased GCF HIF-1α levels in both chronic and aggressive periodontitis patients. This infers that HIF- 1α can be suggested as a potential biomarker in the pathogenesis of periodontal disease.[23] Following scaling and root planing, the salivary HIF-1α levels reduced in group II & III on 30th day and 90th day which could be due to removal of local factors or change in anaerobic environment with decrease in putative organisms and reduction of inflammatory response. At 90th day, comparison of mean difference of HIF-1α between group II & group III was found to be statistically insignificant. In both the groups, there was a drastic reduction in HIF-1α values following non-surgical periodontal treatment and these values were similar to the mean value of group I at 0 day. This was in accordance with the study conducted by **Afacan B et al** who observed that HIF-1α levels was significantly higher in chronic periodontitis patients and was found to be reduced following non-surgical periodontal treatment.[22]

Thus, HIF-1α can be potentially used as diagnostic biomarker for the early detection of periodontal disease as one of the characteristic features of periodontitis also includes hypoxia. HIF-1α, being a highly specific marker for hypoxic changes, it was found to be increased in early periodontal infection which further reduced following treatment. Apart from its diagnostic value, HIF-1α can also be used as a therapeutic tool to assess the clinical improvement in the periodontal parameters post therapy. Further interventional studies with a larger sample size are needed to confirm these results with long term follow-up to explore the role of HIF-1α in different stages of periodontal inflammation.

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CONCLUSION

In the present study, improved levels of salivary HIF-1α were found following periodontal therapy. Low-grade hypoxia or low level of HIF-1 expressed under the normoxic condition in the human periodontium for baseline defense acts as a surveillance “alarm” against significant invasion or periodontitis. Thus, the results of the present study suggest the possible role of hypoxia in pathogenesis of periodontal disease. Further cohort prospective studies are required to authenticate these results.

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