Evaluation of salivary Tumor Necrosis Factor-Alpha in patients with hypothyroidism and periodontitis:  

A Case- Control Study

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Abstract

Introduction: The immune system has served as a bridge among periodontitis and the endocrine system, particularly in the case of thyroid disorders. After non-surgical periodontal therapy for hypothyroid patients, serum thyroid stimulating hormone levels fall within the normal limits, which reduces the inflammatory markers linked to both periodontal and hypothyroid diseases. The pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α), released by macrophages, plays a significant role in bone loss caused by periodontitis. TNF-α will increase the production of collagenases, prostaglandin E2, chemokines and cytokines, cell adhesion molecules, and factors that are related to bone resorption. The aim of our study is to evaluate the salivary level of TNF-α in hypothyroidism patients with periodontitis in comparison to healthy controls.

Material and subject: Saliva were collected from 90 subjects with age ranged between (22-65) years, 50 of them hypothyroid patients (25 patients with hypothyroidism and healthy periodontium, 25 patients with hypothyroidism and periodontitis) and 25 patients with periodontitis and systemically healthy compare with 15 subject as control (systemically healthy with healthy periodontium). Detection of TNF-α in saliva was done by using Enzyme Linked Immunosorbent Assay.
Result: The current results revealed that salivary TNF-α levels are significantly high ($p<0.001$) in diseases group (periodontitis group (132.8 ng/L), hypothyroid patients groups without periodontitis (128.4 ng/L) and with periodontitis (117.0 ng/L) as compared to control group (73.4 ng/L), while there are no significant difference between diseases group.

Conclusion: Salivary TNF-α level increased significantly in all diseased groups (periodontitis group, hypothyroidism groups with and without periodontitis) in comparison to healthy subject.

Key Words: Periodontal disease, Tumor Necrosis Factor-α, Hypothyroidism, Saliva.

1. Introduction

A connection between periodontitis and hypothyroidism was found \(^{(1)}\). To confirm this link, measure the strength of any correlation with sickness severity, show causality, and determine the contribution of each disease to the pathogenesis of the other, additional controlled, prospective clinical and immunologic studies would be required \(^{(2)}\). The cytokines generated because of thyroid dysfunction may have served as the catalyst for an increased inflammatory cascade. This could result in higher local concentrations of inflammatory mediators like cytokines and prostaglandins, increased concentration of matrix metalloproteinase \(^{(3)}\), and other proteinases with damaging consequences on bone and connective tissue, ultimately resulting in osteoporosis and periodontal breakdown \(^{(1)}\).

Periodontal diseases are multifactorial, multi-microbial conditions that gradually destroy the teeth supporting structure, dental biofilm is thought to be a substantial contributor to these disorders, but host immunological responses do the majority of the damage \(^{(4, 5)}\). The host response is thought to be predominantly mediated by neutrophils, monocytes/macrophages, B and T lymphocytes, when these are activated, inflammatory mediators are created, including chemokine’s, cytokines, arachidonic acid metabolites, and proteolytic enzymes. Overall, these contribute to tissue degeneration and resorption of bone by activating a variety of different host degradative enzymes \(^{(6)}\). If periodontitis-related tissue damage is not identified and treated effectively, it can result in the breakdown of both hard and soft tissues and eventually tooth loss \(^{(7)}\).

Thyroxine and triiodothyronine, often known as T4 and T3, respectively, are two important related hormones produced by the thyroid gland. These hormones are important for the growth, development, and metabolism of the majority of vertebrate tissue \(^{(8)}\). As a result, a lack of or abnormally low level of these hormones can cause hypothyroidism, a common endocrine condition with a variety of adverse effects on the body \(^{(9)}\). Hypothyroidism characterized by inability of the thyroid gland to produce enough thyroid hormone to meet the body's metabolic needs; untreated hypothyroidism can cause hypertension, dyslipidemia, infertility,
cognitive deficits, and neuromuscular impairment (10). A clinical case of primary hypothyroidism is diagnosed when the concentrations of TSH are higher than the standard range (>4.5 mIU/L) and a free thyroxine concentration is under the reference range (< 12 pmol/L) (11).

Biomarkers are the characteristics/product of the body at can be used to understand the prognosis, etiology, diagnosis, progression, remission, or outcome of medical treatment (12). Cytokines defined as soluble proteins that bind to specific receptors on target cells and start intracellular signaling cascades, which modify how genes expressed and result in phenotypic changes in the cell (13).

Tumor necrosis factor (TNF-α) is a pro-inflammatory cytokine that regulates cell proliferation, death, and division in addition to inducing the production of other cytokines (14). TNF-α is one of the TNF superfamily's members (15). Although natural killer (NK) cells, T cells, mast cells, and endothelial cells can also create TNF-α, macrophages are primarily the source of TNF-α (16). TNF-α is released in substantial amounts when macrophages come into contact with interleukin-1 (IL-1) or lipopolysaccharide (LPS) (17).

Both endogenous and exogenous thyroid hormones and TSH affect the homeostasis and metabolism of bones (18). It is common knowledge that different hormones cause different reactions in the bones of the skeleton. TNF-α is one of the key cytokines that promote inflammation, is produced locally in a number of tissues under a number of different clinical circumstances, including thyroid dysfunction (18). This cytokine has the ability to stimulate the production of metalloproteinase by the periodontium's resident cells, which degrades connective tissue, as well as the growth and activation of osteoclasts, which degrades alveolar bone (18). Bacteria produce The endotoxins in tooth plaque elaborate with this TNF-α to worsen the inflammatory cascade by inducing the production of other cytokines that activate matrix metalloproteinase and cause periodontal destruction (18).

Saliva is an aqueous solution contains both inorganic and organic components, it is a complex, diluted and colorless solution (19). Saliva offers a novel, non-invasive, and straightforward method to aid in disease diagnosis, saliva collection is comparatively risk-free and reduces the possibility of virus dissemination (20). The structure of the microbiota as well as changes in biochemicals, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and proteins can detected in saliva, which is now thought to be a possible source of biological markers (20). So it is anticipated that saliva may eventually replace serum or urine tests in the diagnosis of diseases (20).

In this study, we examined the relationship between periodontitis individuals and hypothyroidism with the salivary level of TNF-α. The aim of this study was to show the TNF-α salivary level in people with periodontitis and hypothyroidism in comparison to healthy controls.

Hypotheses

Null hypotheses H₀: there is no significant variation of salivary levels of TNF-α in hypothyroidism patients with periodontitis in comparison to healthy control.
Alternative hypotheses $H_1$: there is a significant variation of salivary levels of TNF-$\alpha$ in hypothyroidism patients with periodontitis in comparison to healthy control.

2. Material and Methods

2.1 Study design and ethics

90 Participants

Periodontal Examination (FMPS, BOP, PPD, CAL)  TSH level

Group A: Systemically healthy with healthy periodontium as control group included 15 subjects

Group B: Systemically healthy with periodontitis group included 25 subjects

Group C: Hypothyroidism without periodontitis group included 25

Group D: Hypothyroidism with periodontitis group included 25

Collection of salivary samples

ELISA

Statistical Analysis

Figure A: Consort flowchart of design of the study.
Our study was a case-controlled research that took place from January 2022 to June 2022 at the Teaching Clinics of the Periodontics Department, College of Dentistry, University of Baghdad and the Educational Laboratories, Medical City. The College of Dentistry Ethics Committee at the University of Baghdad reviewed the study and granted it ethical approval (Reference number: 543, Project number: 543522, Date 17/4/2022). In this study, the percentage of male and female as follow male 32%, female 68% with mean age of (39.5).

Healthy periodontium defined as: intact periodontal tissue (no probing attachment loss), probing pocket depth PPD ≤ 3 mm, and bleeding on probing BOP <10% (22). All cases of periodontitis Were defined in reference to (23): Interdentally detectable clinical attachment level CAL at ≥2 non-adjacent teeth or CAL present at ≥3mm on the buccal or lingual/palatal surfaces in conjunction with pocketing >3mm at ≥2 teeth and also PPD ≥5mm or PPD at ≥4mm with BOP. While patients with hypothyroidism defined as: TSH: >4.5 mIU/L, T4: <12 pmol/L, T3: <3 pmol/L (11).

The salivary samples used to compare the salivary levels of TNF-α in-patient with hypothyroidism and periodontitis to healthy controls. Furthermore, in this study we will correlate Clinical parameters assessments to their corresponding biomarker concentration.

2.2 Inclusion and exclusion criteria

Included participants must have a new diagnosis of hypothyroidism, have at least 20 teeth and do not take treatment in the last 3 months. While the exclusion criteria for participant in this study include Subjects who consume alcohol or smoke, have a systemic disease other than hypothyroidism, any patients received extensive periodontal therapy, taking antibiotics, anti-inflammatory drugs, or any medication related to hypothyroidism. Patients that have any inflammatory oral disease that may affect the biomarkers under investigation, women take contraceptive pill, pregnant or nursing women.

2.3 Sample size

To calculate sample size, we use TNF-α as a primary outcome of the study. The concentration of this biomarker during health is estimated to be equal to (2.15pg/ml) and during periodontitis, its concentration is (12.92pg/ml) (17) which gives an odd ratio of (6) between periodontal health and periodontitis. Epitools software used to calculate sample size at 95% confidence interval and 5% error margin. The total sample size is 90 subjects divided into four groups, each case group will consist of 25 subjects and control group will consist of 15 subjects.

2.4 Enrollment of participants

The study's participants will include those seeking periodontal therapy at the Teaching Clinics of Department of Periodontics at the University of Baghdad’s, College of Dentistry as well as those attending the Educational Laboratories in the Medical City. After receiving a thorough description of the study's goals and procedures, every subject will freely sign an informed consent form and Become enrolled in the study.
2.5 Calibrations

Before the start of the study, calibration sessions will perform between the gold-standard periodontics specialist and a main investigator. Kappa-coefficient assay was used to evaluate inter- and intra-examiner calibration for categorical variables (PI and BOP), a kappa value of 75% was the desired level. The level of agreement for continuous variables (PPD and CAL) should be >0.9 as assessed by the Interclass coefficient assay. These sessions will carry out on five patients.

2.6 Periodontal parameters and clinical examination

All teeth, excluding the third molar, will be included in periodontal examination. This includes determining of full mouth plaque score (FMPS) \(^{(24)}\), gingival bleeding on probing (BOP) \(^{(25)}\), probing pocket depth (PPD) \(^{(26)}\), and clinical attachment level (CAL) \(^{(27)}\), using a periodontal probe (Michigan O probe). Thyroid investigation will be done on patients with hypothyroidism (TSH and T4). \(^{(11)}\)

2.7 Collection of salivary samples

Unstimulate Saliva from each patient was taken before any oral examination was performed and placed in sterile test tubes \(^{(28)}\). The expected amount of saliva to be collected per person was 3 ml on average. The obtained samples will immediately put on ice and centrifuged at 1000 rpm for 15 minutes. Then, the clear salivary supernatants will aspirate using a micropipette into a plastic Eppendorf tube. The samples then frozen to -20 c prior to ELISA analysis.

2.8 Enzyme-linked immunosorbent assays Laboratory procedure

After the collection of salivary samples, we allow them to melt at room temperature before beginning the experimental procedures. Then saliva samples will analyze for protein levels of TNF-\(\alpha\) using commercially available ELISA kits that will ordered from MyBioSource in California, USA.

2.9 Statistical analysis

Descriptive statistics including mean, SD, median, and IQR utilized for the continuous data, while frequency and percentage used for the categorical variables. The distribution of parametric data examined using the Shapiro-Wilk test. The Kruskal-Wallis test served as a substitute for non-parametric data. If clinical and biochemical parametric variables were correlated, it was determined using the Spearman's or Pearson's correlation test (depending on the distribution of the data).

3. Result

The age of participants ranged (22-65) years with data (38.97±13.574, 39.50), in this study there were a significant female's predominance among study groups (n=61). Descriptive statistics of clinical parameters (full mouth plaque score FMPS, bleeding on probing BOP, probing pocket depth PPD and clinical attachment level CAL) showed that all clinical parameters had their highest data in patients of group B (FMPS (77.471±23.292, 80.600), BOP(56.262±17.399,57.000), PDD(3.990±1.873,4.560),
When compare the age among groups by using Kruskal-Wallis test (multiple pairwise comparisons) test showed a statistical significant (P<0.05) among study groups (Group A with Group B, Group A with Group D, Group B with Group C, Group C with Group D) as shown in Table 2. Comparison of clinical periodontal parameters (FMPS, BOP) by using Kruskal Wallis test (multiple pairwise comparisons test) the result showed a statistical significant among study groups (Group A with Group D, Group A with Group B, Group C with Group D, Group B with Group C). While for PPD we use Mann-Whitney comparison, the result showed a statistical significant between (Group B and Group D), for CAL T test used, the result showed a statistical significant between (Group B and Group D) as shown in Table 3.

Biochemical tests indicate that the concentration of salivary TNF-α in cases group (Group B (132.8 ng/L), Group C (128.4 ng/L) and Group D (117.0 ng/L)) was higher significantly in comparison to control group (73.4 ng/L). While TNF-α level had a non-significant difference between cases group (Figure A). When we compare the level of TNF-α among study groups statistically by using Kruskal Wallis test (multiple pairwise comparisons), the result indicate that there is a statistical significant (P<0.05) among study groups (Group A with Group B, Group A with Group C, and Group A with Group D) as shown in table 5.

When levels of TNF-α were correlated with the clinical periodontal parameters by using spearman correlation coefficient because the data was not normally distributed, the results showed a positive significant correlation of TNF-α with BOP (r=0.545**, p=0.005) and PPD (r=0.430*, p=0.023) in-group D. While TNF-α had a non-significant negative correlation with FMPS (r=-0.567, p=0.027) and BOP (r=-0.215, p=0.441) in-group A and in-group C, FMPS (r=-0.105, p=0.619), BOP (r=-0.043, p=0.837).

In addition, there were a non-significant positive correlation among TNF-α and all clinical periodontal parameters in-group B {PI (r=0.133, p=0.527), BOP (r=0.334, p=0.103), PPD (r=0.059, p=0.780), CAL (r=0.385, p=0.057) and with FMPS (r=0.184, p=0.379) and CAL (r=0.302, p=0.143) in-group D (Table 4).

4. Discussion

Salivary TNF-α showed high level in all diseased groups as inflammatory biomarker in comparison to healthy control. However salivary TNF-α did not show a significant level in hypothyroidism when comper to periodontitis and healthy individuals. This study was conducted to evaluate the level of TNF-α in hypothyroidism and periodontitis patients in comparison to healthy control.

Clinical definition of periodontitis is not enough for detection of this disease, the definitions of periodontitis based on marginal radiographic bone loss are limited because they are not specific enough and fail to detect mild to moderate periodontitis (29). In addition,
the definitions of periodontitis based on radiographic bone loss should be restricted to the stages of mixed dentition and tooth eruption (30). For most patients, clinical parameters are effective instruments for evaluating their health and disease states. However, other individuals due to multiple factors more likely to develop and maintain a dysbiotic microbiota in conjunction with chronic periodontal inflammation and it is unclear whether the clinical parameters used today are adequate to monitor the progression of the disease so biomarkers are currently available (23). Biomarkers are predicted to make significant contributions to a better assessment of the grade of periodontitis and to increased diagnostic accuracy in the early diagnosis of the disease, these might help to assist the periodontitis grading and staging (23).

We choice saliva in this study because salivary sample analysis is a cost-effective strategy since it is completely noninvasive and requires little effort from the patient, it is simple to collect, store, and transmit the samples (31). Saliva is a rich source of readily available proteins and peptides that can be used to evaluate biomarkers generated during the onset and course of disease (32). The focus of current research has been on saliva-based diagnostics because there are linkages between many systemic diseases and the salivary molecules have good correlations with blood levels (33).

In this study, the result showed that the severity of periodontal disease increase with age progression in periodontitis group and hypothyroidism with periodontitis group. This may related to the fact that aging negatively affects innate and adaptive immune response cells and molecules, including in the oral cavity (34). Also for hypothyroidism, almost all bodily tissues, at all stages of development, depend on thyroid hormones to function properly (35). Other study related this to an increase in the synthesis of anti-thyroglobulin antibodies, anti-peroxidase antibodies, and TSH hormone (36).

Salivary level of TNF-α increase from health to disease, in which the lowest concentration founded in control group while the highest concentration founded in cases group, for periodontitis patients the TNF-α, is a one of the first signaling cytokines, which are also common in periodontal lesion. TNF-α can be recognized as an essential immune response mediator in periodontitis as it is directly produced by a variety of cells and typically activates the immune system by secondary mediator molecules, inducing chemokine’s, adhesion molecules, and prostaglandin E2 (37, 38).

The release of matrix metalloproteinase, stimulation of osteoclast differentiation, elevation of phagocytic and neutrophil activity, and induction of apoptosis in fibroblasts are all effects of TNF-α. The amount of TNF-α is elevated in plaque-induced inflammation, such as that linked to chronic periodontitis (37, 38).

Also in hypothyroidism groups both with and without periodontitis the TNF-α level was high in this study, this agreed with the result of other study that showed high level of TNF-α in hypothyroid patient in comparison to healthy subject (1). TNF-α is produced locally in several tissues in a variety of clinical conditions, including thyroid dysfunction (18).

For correlation of salivary TNF-α with clinical periodontal parameters the result showed that there were a
Significant positive correlations of TNF-α with BOP and FMPS in-group of hypothyroidism with periodontitis. The positive significant correlation of TNF-α with BOP and FMPS may be due to the fact that the TNF-α is known as a pro-inflammatory cytokine that affects the activation of inflammatory leukocytes and change of vascular permeability (39). Also the positive correlation among the level of the TNF-α and the disease severity was mightily confirm the hypothesis that this cytokine are likely to be involved in the periodontitis pathogenesis (40).

The limitation of this study was small sample size. Use saliva instead of serum for detection of cytokines level because the serum has the bulk of cytokines concentrations spatially in systemic disease. We did not include a group of gingivitis to examine the level of cytokines in mild inflammation; also, we did not classify the periodontitis according to extent and severity as stage and grade. We examine the level of TSH and T4 as a diagnostic tool for hypothyroidism, but we did not compere the level of them with the level of biomarkers. Monitoring the level of the selected salivary TNF-α following periodontal therapy will provide a clear picture. Suggestions include need larger sample size. Examine the level of RANKL with OPG to obtain the OPG/RANKL ratio. Use serum instead of saliva for estimation the level of biomarkers especially in systemic diseases. Compare the levels of TSH and T4 with the salivary levels of OPG and TNF-α in hypothyroidism patients. Examine the level of TNF-α after periodontal therapy. Gingivitis group Included in the study. Classify periodontitis according to severity and extent as stage and grade.

5. Conclusion

Salivary TNF-α level increased significantly in all diseased group (periodontitis group, hypothyroidism groups with and without periodontitis) in comparison to healthy subject, but there are no difference in level of salivary TNF-α between diseased group.

Conflicts of Interest

The authors declare that they have no conflicts of interest.
**Tables:**

Table 1: Statistical description for sex, age and clinical periodontal parameters among study groups.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Frequency(n)</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent (%)</td>
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<td>1</td>
<td>15</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>%</td>
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<td>40%</td>
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<td>60%</td>
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</tr>
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<td>15</td>
<td>24</td>
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</tr>
<tr>
<td></td>
<td>%</td>
<td>80%</td>
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<td>96%</td>
<td>40%</td>
<td>68%</td>
</tr>
<tr>
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<td>25</td>
<td>25</td>
<td>25</td>
<td>90</td>
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<tr>
<td>Age</td>
<td>Mean±SD</td>
<td>23.53±3.091</td>
<td>45.48±9.319</td>
<td>29.12±5.769</td>
<td>51.56±9.917</td>
<td>38.97±13.574</td>
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<tr>
<td></td>
<td>Median</td>
<td>22.00</td>
<td>42.00</td>
<td>30.00</td>
<td>46.00</td>
<td>39.50</td>
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<tr>
<td>Clinical periodontal parameters</td>
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<td></td>
<td></td>
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<tr>
<td>FMPS</td>
<td>Mean±SD</td>
<td>5.860±1.725</td>
<td>77.471±23.292</td>
<td>7.740±1.779</td>
<td>64.280±23.390</td>
<td>42.502±36.573</td>
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<td>Median</td>
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<td>38.500</td>
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<tr>
<td>BOP</td>
<td>Mean±SD</td>
<td>4.586±1.481</td>
<td>56.262±17.399</td>
<td>7.272±1.752</td>
<td>49.840±14.226</td>
<td>32.257±26.277</td>
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<tr>
<td></td>
<td>Median</td>
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<td>57.000</td>
<td>7.500</td>
<td>50.000</td>
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<tr>
<td>PPD</td>
<td>Mean±SD</td>
<td>0.000±0.000</td>
<td>3.990±1.873±</td>
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<td>2.984±2.109</td>
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<tr>
<td>CAL</td>
<td>Mean±SD</td>
<td>0.000±0.000</td>
<td>3.640±0.803</td>
<td>0.000±0.000</td>
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<td>3.540</td>
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</table>
Table 2: Multiple pairwise comparison of age among study groups.

<table>
<thead>
<tr>
<th>Group pairs</th>
<th>Group A-Group C</th>
<th>Group A-Group B</th>
<th>Group A-Group D</th>
<th>Group B-Group C</th>
<th>Group C-Group D</th>
<th>Group B-Group D</th>
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</thead>
<tbody>
<tr>
<td>P value</td>
<td>1.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.920</td>
</tr>
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</table>

* The significance level is 0.05

Table 3: Multiple pairwise comparison of (PI, BOP) among study groups, Mann-Whitney comparison of PPD between group B and group D, T test comparison of CAL between group B and group D.

<table>
<thead>
<tr>
<th>Clinica Parameters</th>
<th>Group pairs</th>
<th>Group A-Group C</th>
<th>Group A-Group D</th>
<th>Group B-Group C</th>
<th>Group C-Group D</th>
<th>Group B-Group D</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMPS</td>
<td>P value</td>
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<tr>
<td>BOP</td>
<td>P value</td>
<td>0.499</td>
<td>0.000</td>
<td>0.000</td>
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<td>1.000</td>
</tr>
<tr>
<td>PPD</td>
<td>P value</td>
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<td>0</td>
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<td>0.018</td>
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<tr>
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<td>P value</td>
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*The significance level is 0.05

Table 4: Correlation between TNF-α and clinical periodontal parameters among study groups.

<table>
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<tr>
<th>Groups</th>
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<th>BOP</th>
<th>PPD</th>
<th>CAL</th>
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<tbody>
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<td></td>
<td></td>
<td>R</td>
<td>r</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Group A</td>
<td>TNF-α</td>
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<td>0.027</td>
<td>-0.215</td>
<td>0.441</td>
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<tr>
<td>Group B</td>
<td>TNF-α</td>
<td>0.133</td>
<td>0.527</td>
<td>0.334</td>
<td>0.103</td>
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<tr>
<td>Group C</td>
<td>TNF-α</td>
<td>-0.105</td>
<td>0.619</td>
<td>-0.043</td>
<td>0.837</td>
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<tr>
<td>Group D</td>
<td>TNF-α</td>
<td>0.184</td>
<td>0.379</td>
<td>0.545**</td>
<td>0.005</td>
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</table>

* spearman correlation coefficient
Table 5: Multiple pairwise comparison of TNF-α among study groups

<table>
<thead>
<tr>
<th>Group pairs</th>
<th>Group A-group B</th>
<th>Group A-group C</th>
<th>Group A-group D</th>
<th>Group C-group D</th>
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<th>Group C-group B</th>
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</thead>
<tbody>
<tr>
<td>P value</td>
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<td>1.000</td>
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</table>

The significance level is 0.05

**Figures:**

**TNF-α Distribution**

![TNF-α Distribution](image)

Figure A: Salivary concentration of TNF-α among study groups. The TNF-α level was significantly * higher in patients of cases group than control group, while there were non-significant differences among disease groups.
Reference
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