The Role Of TNF-A in Saliva and Gingival Fluid in Patients with Chronic Periodontitis and Type 2 Diabetes Mellitus

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Abstract

To detect the change of TNF level in saliva and gingival fluid at different time intervals and its association clinical attachment level (CAL) in patients with chronic periodontitis (CHP) and T2DM (type 2 diabetes mellitus). 40 patients aged 35 - 60 years with CHP and T2DM participated in the study. They were all treated conservatively and in addition with low level laser therapy (LLLT) (660 nm, 10mW, 8 min / day in 5 consequent ssessions). T2DM patients have controlled their increased blood sugar with oral administration of 750 mgr Glucophage XR tablets, 2 x per day. Conservative treatment included removal of supragingival tartar and root scaling and planing in all dental quadrants.TNF-α was determined in saliva and gingival fluid at the first treatment, 6 weeks and 3 months after treatment. Significant difference between the values of TNF-α in saliva at first examination, six weeks and three months after therapy p <0.001. TNF-α values in the gingival fluid after 3 months p <0.001 were significantly lower than the values at 6 weeks. At the first examination, the CAL values were in the interval 5.28 ± 0.17 mm, after 6 weeks 4.69 ± 0.19 mm, after 3 months 4.48 ± 0.20 mm. Correlation between CAL and TNF-α in the saliva at first examination R = 0.09, (p> 0.05), 6 weeks after treatment R = 0.11, (p> 0.05) and after 3 months R = 0.12, (p> 0.05) showed weak positive insignificant correlation. Correlation between CAL and TNF-α in the gingival fluid at the first examination showed weak negative insignificant correlation R = -0.09 (p> 0.05), after 6 weeks Spearman Rank R= -0.22 (p> 0, 05) showed moderate weak negative insignificant correlation, and after 3 months weak positive insignificant correlation R = 0.08 (p> 0.05).TNF-α values in saliva and gingival fluid tend to decrease 6 weeks and 3 months from the treatment compared to the results from the first examination. Weak positive insignificant correlation between TNF-α values in saliva, GCF, and CAL after 3 months from the treatment was confirmed.

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Introduction

Chronic periodontitis (CHP) as a progressive disease induced by dental plaque bacteria resultes in gradual destruction of the periodontal tissues. In its etiopathogenesis it is associated with many systemic diseases: cerebral[1], pulmonary[2], and cardiovascular, endocrine[3] etc. They all act as comorbidities and contribute to destruction of the periodontium that leads toward tooth loss. [4] Hence, patients diagnosed with diabetes are at even greater risk for maintaining an optimal oral health and a healthy periodontium. Low glycemic values may worsen periodontal status in patients with chronic periodontitis but smoking has been shown to have some effect on periodontal damage in patients with diabetes and with chronic periodontitis. [5]

A higher rate of emotional disturbances has been reported [6], the positive correlation of GCF with periodontal support becoming negative, i.e. there is a transition of certain types

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of candida from commensals to pathogenic microorganisms[7] which may affect the course, evolution and severity of periodontal disease.

Possible association of chronic diseases with periodontal disease is indicated by the host's immune response, which is believed to be crucial for the course, progression, and severity of the disease.[8]

Previous experience suggests that T2DM is probably among greatest risks for initiation and progression of the periodontal disease because it alterates the delicate balance between periodontal tissues thus favoring the process of the periodontal destruction. Disturbed balance in the periodontal tissues may emphasize the two-way link between periodontal disease and other chronic systemic diseases. In these conditions we emphasize the key role of the pro- inflammatory cytokines as IL-1, TNF α, IL-6, IL-17 in saliva and the gingival fluid and their variables in status of health and disease.[9,10] IL-1β, TNF α, IL-6 are released from polymorphonuclear leucocyte cells in response to inflammatory and infectious stimuli. [11–13]

Experimental conditions have shown that papaya seed extract (Carica papaya linn) can reduce IL-1β levels in patients with periodontitis, thanks to ethanol extract which has anti-inflammatory potential [14], while papaya leaves have biolarvicidal effects against Aedes aegypti. [15]

TNF α as important pro-inflammatory cytokine and immune modulator affects the recruitment of inflammatory cells, bone mass resorption, stimulates the IL-1, affect the differentiation of osteoclasts by activation of macrophages. [16] Macrophages and neutrophil leucocytes are not the only ones that stimulate the production of TNF α. It may respond to stimulation of adipocytes, keratinocytes, fibroblasts, natural killer (NK), T and B cells. Activation of TNF α is the cause of bone resorption through induction of osteoclasts proliferation and extracellular matrix production. It also stimulates the production and activity of matrix metalloproteinases, other cytokines, collagenases, and prostaglandins. [17, 18]

Based on these facts, it is quite clear that changes in TNF α values affect the overall health status of the individuals (systemic and oral).

Dental plaque accumulations rich in microorganisms stimulate TNF α production and emphasize its pathological effects, among other it affects the clinical attachment level (CAL), periodontal pockets depth (PD) and worsens clinical manifestation of the chronic periodontal disease.

It would be logical if correction of TNF α values result in stabilization of the periodontal degradation thus slowing the pathological events in the periodontal tissues. Therefore, conservative therapy in the treatment of periodontitis would be an imperative.[19]

Reduction of the periopathogens is undoubtedly important in the treatment of this very common disease. However, events in the oral cavity in patients with T2DM conduct slightly different circumstances in terms of the immune and fitness capacity of the individuals.

Chronic infections are known to be potential risk factors for diabetes mellitus and suggest a link between CPD and T2DM, although the mechanisms of this association still remain unclear.[20]

In addition to the commonly used conservative periodontal treatment, increased focus is given to the application of low level laser therapy (LLLT) as an adjuvant to the initial therapy.

Numerous biostimulating effects of LLLT are emphasized, such as the impact on inflammation and circulation, the healing of lesions. Correction of the clinical periodontal parameters is only a segment from the numerous effects that have been proven so far.[21-24]

Based on these known facts, we set our goal to track TNF α changes in the saliva and the gingival fluid at different time intervals and to register its possible association with CAL in patients with T2DM and CPD.

Materials and methods

In the study 40 patients with CHP were included, they were diagnosed with T2DM previously. Respondents were selected by random from the Department of Periodontics and Oral Diseases at the University Dental Clinical Center in Pristina, Kosovo.

First, patients were informed about their participation in the study and they all gave their written consent. Prior to the survey conduction, a request with an explanation and protocol of the research was submitted to the Ethics Commission.

The research received a positive opinion and approval for the realization of the study. Group of 40 patients of both sexes, aged 35-60 years were included in the study. After diagnosis for CPD, conservative periodontal treatment in addition with LLLT was performed.

All subjects have controlled their increased blood sugar level with an oral administration of 750 mgr Glucophage XR tablets. 2x per day (Merck Sante, France). Research procedure was fully implemented by the same one practitioner.

Patients were excluded from the study if they were: treated with antibiotics in the previous 4 months, pregnant, smoke cigarettes, with on-going systemic disease, treated with immunosuppressive drugs, medications that affect periodontal status as phentoine, cyclosporine, calcium channel blockers etc.
Patients were included in the study after they were: diagnosed with diabetes mellitus type 2, regulated the diabetes with oral antidiabetics, diagnosed for periodontitis with 4 mm depth of the periodontal pockets in at least 50% of the affected teeth.

At the first examination, a diagnosis was made by clinical examination and retroalveolar and panoramic imaging. The measurement of clinical loss of attachment (CAL) was performed with periodontal probe type PCPUNC 156, HU-Friedy, Chicago, IL USA. Measurements were performed in three stages. At the base-line (first examination), 6 weeks and 3 months after the treatment.

After the diagnostic segment, all patients underwent the initial phase of therapy, ie. conservative treatment (removal of soft and hard deposits, root planing and scaling, and deep rinsing of the periodontal pockets with 1% chlorhexidine solution. This procedure was carried out in all 4 quadrants, in 5 sessions, including ultrasonic cleaning of supragingival tartar by SONICflex ™ quick 2008 / L, KAVO, Germany. The root planning was done with Grace's Curetes, model Hu-Friedy, Chicago, IL, USA. All patients were instructed to maintain daily oral hygiene.

After the conservative treatment, LLLT was applied, five days in a row, performed with 660 nm, 10mW, 8 min / day, in contact with gingiva; laser model: Hager & Werken LASER HF "confort" V023-17, Duisburg, Germany.

All subjects were assessed for TNF-α values in saliva and gingival fluid at first treatment –baseline and then at 6 weeks and 3 months after the treatment.

Index of apical epithelial migration, ie. Clinical Attachment Level (CAL) has been determined (AAP1999). CAL was determined with periodontal probe measured from the enamel-cemental junction to the bottom of the periodontal pocket.

1. Pre-treatment and saliva collection

Collection of samples (general procedure)

Before collecting samples patients were informed that they should follow given recommendations to:

• avoid foods with high values for sugar, acidity, or caffeine just before sample collection as they may compromise the test by lowering saliva pH value and alter the bacterial flora;
• avoid alcohol, caffeine and nicotine as well as medications during the previous 12 hours;
• refrain from consuming a heavy meal 60 minutes before collecting saliva;
• avoid vigorous physical activity 4-6 hours before collecting the material;

Before the procedure, each patient rinsed their mouth with water in order to remove food debris and waited for 10 minutes after rinsing to avoid sample dilution.

The procedure for determining TNF alpha (TNF α) started with saliva extraction.
Samples of non-stimulated saliva in the examined group, in the amount of 5-10 cm^3, were collected in the morning before breakfast. The non-stimulated saliva in sterile special cups was distributed to a biochemical laboratory, where samples were stored at -20 ° C until the analysis was performed. During the collection, patients spated on every 30 seconds and collecting period have lasted for 5 min.

After collecting the sample, the time and date of collection of samples were recorded for each individual of the examined group. All samples that had visible blood contamination were excluded from the research.

Before the start testing (analysis), samples were brought to room temperature (18-25 ° C), then centrifuged for 20 minutes at 1000 rpm at 2-8° C. Supernatant was used for further analysis.

Procedure for determining Tumor necrotizing factor α (TNF-α) from the gingival fluid:
Determination of TNF-α begins with extraction of

gingival fluid. Gingival fluid samples were collected using 2x5mm methylcellulose strips. (Whatmann 3mm Chromatography paper) from at least of 10 periodontal pockets with a depth of ≥4 mm.

In order to minimize the possible contamination with saliva that may interfere with the performance of the analysis, it is necessary to provide a dry working field. For this purpose, before collecting samples, sterile gauze is used to isolate the area around the periodontal pockets and to dry the marked area with an air jet. Using tweezers, the methylcellulose tape is gently and carefully inserted into the periodontal pocket and left there for 30 seconds. This way the gingival fluid enters the paper by capillary forces. After the time has elapsed, the sample tape is transferred to Eppendorf test tubes for micro centrifugation. To estimate the volume of gingival fluid collected during sampling, Eppendorf test tubes are measured after the sample has been collected. The difference in mass between the empty Eppendorf test tube and the Eppendorf test tube containing the collected sample is equal to the volume of the collected gingival fluid. The collected samples can be analyzed immediately or stored until the analysis, frozen at a temperature of - 20 ° C. The samples were diluted in a 4: 5 ratio (50 µg) and incubated. The resulting complexes were washed, incubated and detected. Definitive values of TNF-α in the gingival fluid obtained by software application are expressed in picograms for 30 sec. (pg / 30 s).

Determination of TNF-α in saliva and gingival fluid

In the test procedure, reagents were prepared first, followed by the preparation of a standard reagent. Seven tubes containing a standard 1.0 µL diluent were used to make a double dilution series according to the image shown below. Washing buffer was diluted with 30mL of concentrated wash buffer in 750mL wash buffer with deionized or distilled water. The procedure continued with biotinylated Detection Ab, followed by determining the concentrated HRP Conjugate and dissolving the solution with the concentrated HRP Conjugate to the working concentration using the Concentrated HRP Conjugate Diluent (1: 100).

A test protocol was performed, adding 50 µL of Stop Solution in each well. The color immediately turns yellow. The optical density (OD value) of each well was determined simultaneously, using a 450 nm microplate tile reader, and the results were finally calculated.

The findings were compared at the first examination, 6 weeks and 3 months after CAL and LLLT treatment.

Statistical processing and data analysis:

Statistical program Statistic 7.1 for Windows was used. Differences in the analyzed parameters from the first examination, after 6 weeks and after 3 months from the treatment, were tested using Friedman ANOVA Chi Sqr. / p; Correlation in the analyzed individual relations CAL and TNF-α, in saliva and gingival fluid in the first examination, after 6 weeks and 3 months from the treatment was analyzed using Spearman Rank R (R). The data were displayed in tables and graphs.

Results

TNF-α in saliva ranged from 131.85 ± 5.51 pg/ml, after 6 weeks from therapy it ranged from 126.12 ± 4.02 pg/ml, and after 3 months it oscillated within 119 , 81 ± 4.05 pg / ml. Among the values of TNF-α in saliva (first examination, six weeks and three months after therapy) for Friedman ANOVA Chi Sqr. (N = 40, df = 2) = 80.00 and p <0.001 (p = 0.000) there was a significant difference.

In the gingival fluid at the first examination TNF-α in the gingival fluid varied in the interval 31.74 ± 0.68 pg/30 s, after 6 weeks it varied in the interval 21.84 ± 0.60 pg/30 s, and after 3 months from the treatment varied in the interval is 20.49 ± 0.36 pg /30 s.

Among TNF-α values in gingival fluid (first examination, six weeks and three months after therapy) for Friedman ANOVA Chi Sqr. (N = 40, df = 2) = 79.04 and p <0.001 (p = 0.000) there was a significant difference (Table 1).

TNF-α	Average Rank	Sum of Ranks	Mean	Std.Dv.
saliva				
First examination	3,00	120,00	131,85	5,51
After 6 weeks	2,00	80,00	126,12	4,02
After 3 months	1,00	40,00	119,81	4,05
Gingival fluid				
First examination	3,00	120,00	31,74	0,68
After 6 weeks	1,98	79,00	21,84	0,60
After 3 months	1,03	41,00	20,49	0,36

Table 1.TNF-α in saliva and gingival fluid in patients with CHP and T2DM at the examined time intervals.

Among the values of TNF-α in saliva (first examination, six weeks and three months after treatment) for Friedman ANOVA Chi Sqr. (N = 40, df = 2) = 80.00 and p <0.001 (p = 0.000) there was a significant difference.

The value of TNF-α in gingival fluid after 3 months from treatment, for Z = 5.37 and p <0.001 (p = 0.000) was significantly lower than the value at 6 weeks from treatment (Table 2).

TNF-α in saliva	Average Rank	Sum of Ranks	Mean	Std.Dv.
First examination	3,00	120,00	131,85	5,51
After 6 weeks	2,00	80,00	126,12	4,02
After 3 months	1,00	40,00	119,81	4,05
TNF-α in gingival fluid				
First examination	3,00	120,00	31,74	0,68
After 6 weeks	1,98	79,00	21,84	0,60
After 3 months	1,03	41,00	20,49	0,36

Table 2. Differences between TNF-α values in saliva and gingival fluid in group A at different time intervals.

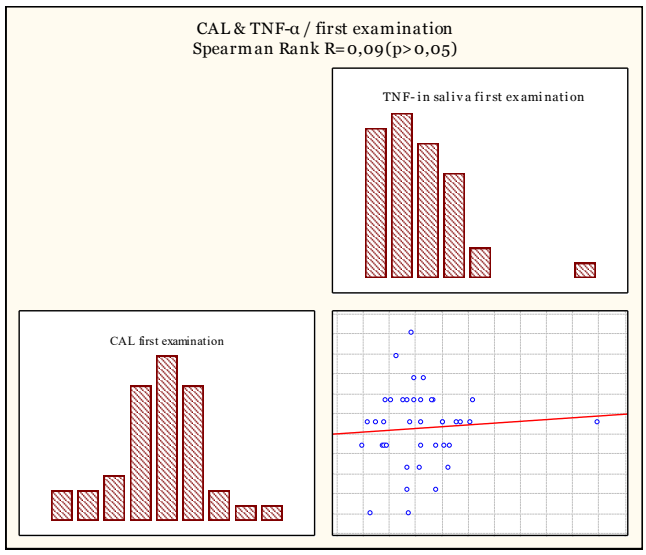
Descriptive statistics of CAL. At the first examination CAL values varied in the interval 5.28 ± 0.17 mm. After 6 weeks from therapy the values were 4.69 ± 0.19 mm. And 3 months from therapy registered values were o4.48 ± 0.20 mm (Table 3).

Index of apical epithelial migration	N	Mean	Confidence -95,00%	Confidence +95,00%	Minimum	Maximum	Std.Dev.
First examination	40	5,28	5,22	5,33	4,90	5,70	0,17
After 6 weeks	40	4,69	4,63	4,75	4,30	5,40	0,19
After 3 months	40	4,48	4,41	4,54	4,10	5,10	0,20

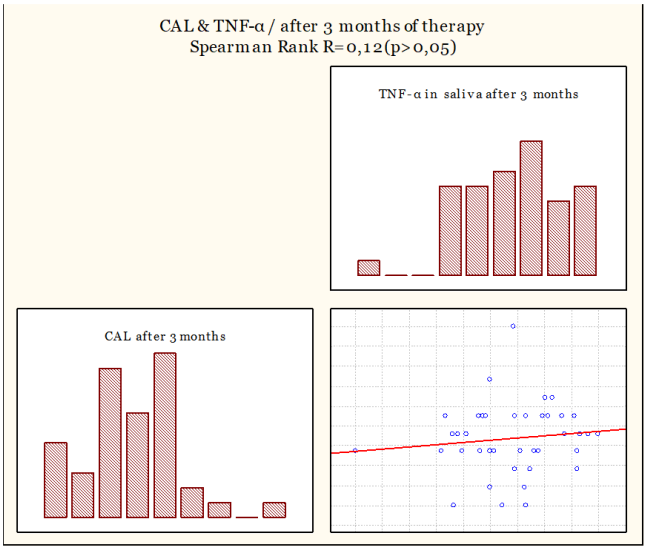
Table 3. Index of apical epithelial migration in group A patients at different intervals.

Correlation between CAL and TNF-α in saliva in patients treated conservatively with additional LLLT at first examination (base line), after 6 weeks and 3 months from the treatment.

Examination of the relationship between CAL and TNF-α in saliva at first examination for Spearman Rank R = 0.09 (p> 0.05) showed a weak positive insignificant correlation (Graph 1), 6 weeks after treatment between CAL and TNF-α, in saliva after 6 weeks from the treatment for Spearman Rank R = 0.11 (p> 0.05) showed weak positive insignificant correlation (Graph 2), while the ratio between CAL and TNF-α in saliva after 3 months from treatment for Spearman Rank R = 0 , 12 (p> 0.05) showed a weak positive insignificant correlation (Graph 3).



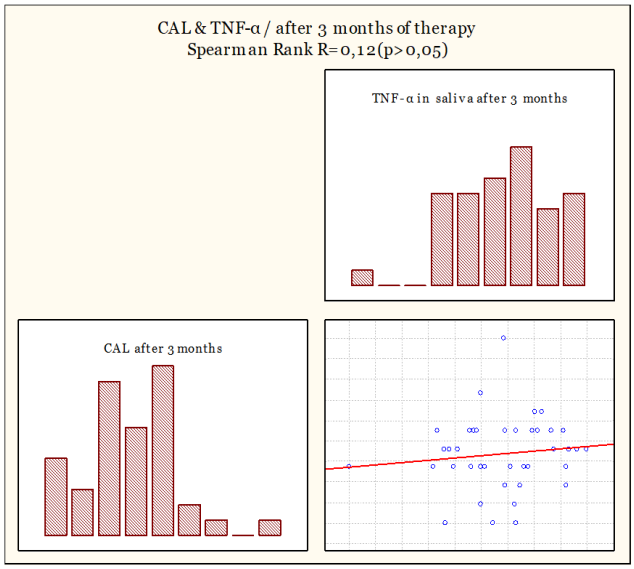
Graph 1. Correlation between CAL and TNF-α in patients treated conservatively with additional LLLT application: at first examination (base line).



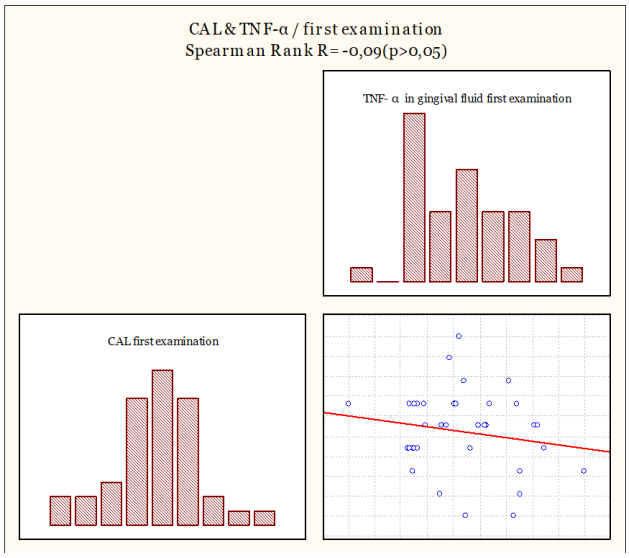
Graph 2. Correlation between CAL and TNF-α in patients treated conservatively with additional LLLT application: after 6 weeks from treatment.

Correlation between CAL and TNF-α in gingival cervical fluid in patients treated conservatively with additional application of LLLT at first examination, after 6 weeks and 3 months from treatment is presented on graphs 4, 5 and 6. Examination of CAL and TNF-α in gingival fluid at first examination for Spearman Rank R = -0.09 (p> 0.05) showed a weak negative insignificant correlation (Graph 4) between CAL and TNF-α in gingival fluid after 6 weeks from therapy for Spearman Rank R = -0.22 (p> 0.05) showed a moderately weak negative insignificant correlation (Graph 5) between CAL and TNF-α in

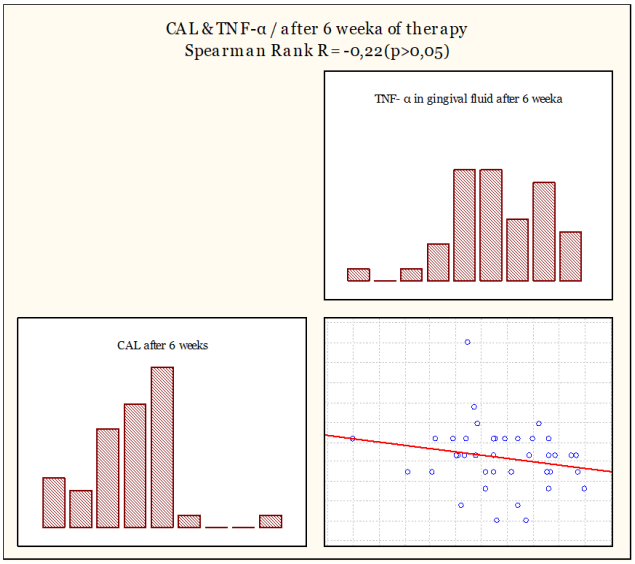
gingival fluid after 3 months from therapy for Spearman Rank R = 0.08 (p> 0.05) showed a weak positive insignificant correlation (Graph 6).



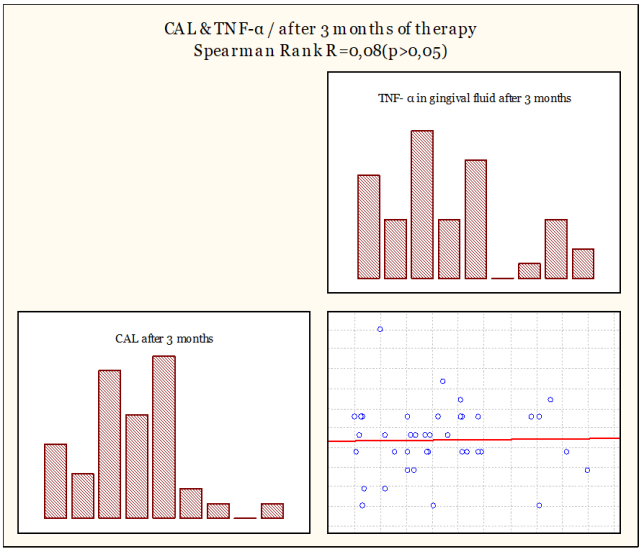
Graph 3. Correlation between CAL and TNF-α in patients treated conservatively with additional LLLT application: 3 months from treatment.



Graph 4. Correlation between CAL and TNF-α in gingival cervical fluid in patients treated conservatively with additional LLLT application: at first examination (base line).



Graph 5. Correlation between CAL and TNF-α in gingival cervical fluid in patients treated conservatively with additional LLLT application: after 6 weeks from treatment.



Graph 6. Correlation between CAL and TNF-α in gingival cervical fluid in patients treated conservatively with additional LLLT application: 3 months from treatment.

Discussion

Diabetes mellitus is a serious disease with the possibility of many complications, including endangering the life of the patient, while on the other side, periodontitis is a chronic progressive disease, which together with caries are the most common diseases in the oral cavity. The mutual influence is indisputable and has been proven many times so far. [25-28]

The two diseases together in one individual create a closed circle for two-way connection which results in worsening of diabetes, and with progression of the periodontal disease. [25] When periodontal infection values of IgG, IgM, are increasing, and the production of anti-KL (cardiolipin) antibodies are growing, which are stimulated through the mechanism of molecular mimicry. [29] Pathological events in the tissues (enzymes or inflammatory mediator) lay at the base of any clinical manifestation of the disease. [30, 31] TNF-α is a proinflammatory cytokine responsible for destruction of the bone substrate, therefore initiating the progression of the periodontal disease.[32] T2DM is believed to cause local destruction of the alveolar bone which affects the periodontal status.[33]
It has been linked to gingival inflammation, increased depth of periodontal pockets, and advanced and accelerated apical epithelial migration. It is proven that TNF-α concentrations are variable in different health conditions, and vary in saliva and gingival crevicular fluid (GCF). Outside the reference values, increased or decreased values for TNF-α affect the periodontal tissue, especially the bone mass. [34] Kurniati et all proved that the level of TNF-α in gingival cervikular fluid (GCF) was significantly higher in patients with diabetes mellitus. [35]

In this study, TNF-α values in saliva and gingival fluid decreased linearly after 6 weeks (126.12 ± 4.02 pg / ml); (21.84 ± 0.60 pg / 30s), and 3 months (119.81 ± 4.05 pg / ml); (20.49 ± 0.36 pg / 30 s) from treatment, compared with the values from the first examination (131.85 ± 5.51 pg / ml); 31.74 ± 0.68 pg / 30s, where for Friedman ANOVA Chi Sqr. (N = 40, df = 2) = 80.00 and p <0.001 (p = 0.000) significant difference was registered.

Variation in TNF-α values in different media is possible in healthy individuals [36], smokers[5,37] or in individuals with systemic disorders including rheumatoid arthritis [38], lupus erithematodus[39], chronic obstructive pulmonary disease[40], diabetes[20,25, 33, 41], psoriasis [42] etc.
When T2DM is issue, elevated levels of TNF-α cause insulin resistance in adipocytes, resulting in impaired insulin signaling through serine phosphorylation. The ultimate effect of these developments is disease progression. [43]

As a local medium, saliva is easily accessible for diagnostic or prognostic purposes, and is a fairly useful indicator through its biocollections it can be used to assess the extent of periodontal damage. Different inflammatory molecules produced in the periodontium are flowing trough the mucosal epithelium and GCF [44] so they can be detected in saliva. Teke [45] confirmed that severity of the periodontal disease progresses with deterioration of metabolic control. However, the importance of salivary levels of TNF-α in pathogenesis of the periodontal disease and other diseases has been unquestionably proven to be, i.e. periodontal disease and diabetes have a synergistic effect and affect the increased values of certain cytokines including TNF-α in GCF. [46-48] These facts would logically follow that periodontal destruction would be controlled under the conditions of regulated diabetes. [45] conservative treatment [49-50] or LLLT[51-52], affect the correction of clinical parameters and TNF-α values in saliva and GCF. We have found similar results with the above-mentioned authors. Our findings coincide with the aforementioned authors [49-52].

Namely, in this research, the examined relationship between CAL and TNF-α in saliva at the first examination (R = 0.09 (p> 0.05), after 6 weeks from treatment for R = 0.11 (p> 0.05) and after 3 months from therapy for Spearman Rank R = 0.12 (p> 0.05) showed a weak positive insignificant correlation.

In the gingival fluid at the first examination, a weak negative insignificant correlation for R = -0.09 (p> 0.05) was shown, after 6 weeks a moderately weak negative insignificant correlation was registered, ie. R = -0.22 (p> 0.05), while after 3 months from treatment for Spearman Rank R = 0.08 (p> 0.05) a weak positive insignificant correlation was recorded.
The results of this study are due to applied conservative treatment in patients with CHP and T2DM. Elimination of the pathological content from the periodontal pockets together with the applied LLLT, reduces the inflammation of the periodontium, by reducing the level of TNF-α values in saliva and in the GCF, which definitely corrects CAL after 6 weeks, and especially after 3 months.

Examinations by individual authors confirm the findings from this study, adding that LLLT as well as affecting cytokine values it has a strong effect on growth factors and proinflammatory mediators.[53] Accelerating epithelial cell proliferation and angiogenesis favorably affects the clinical parameters of the

periodontium. [54]

LLLT also stimulates fibroblast growth thus confirming its positive therapeutic effect. [55] LLLT treatment accelerates microcirculation, resulting in changes in the hydrostatic pressure, resorbs and removes accumulated metabolites, and has anti-inflammatory and anti-edematous effect.[56] Screening patients with diabetes, regardless of the value of blood glucose is an important procedure that should be promptly implemented because of the inevitable association with parodontopathy. [57] In these conditions the approach to the disease of the individual is essential because patients perceive the severity of the disease tend to more solid control [58], regulating the values of CRP in T2DM, which conditions the significant improvements of periodontal health in patients with diabetes. [59]

Conclusions

There is a significant difference between TNF-α values in saliva and gingival fluid (first examination, six weeks, and three months after treatment). After 3 months values are significantly reduced compared to those at 6 weeks. Study has confirmed a weak positive insignificant correlation between TNF-α values in saliva, GCF, and CAL after 3 months from the treatment.

Declaration of Interest

The authors report no conflict of interest.

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