Salivary Levels Of Interleukin-1beta, Tumour Necrosis Factor-α, And C-Reactive Proteins In Smokers Patients With Severe Chronic Periodontitis

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ABSTRACT

Background. Chronic periodontitis(CP) is disease that causes loss of the supporting structures of teeth, cementum, alveolar bone and periodontal ligaments. Cytokines are important mediators of immune responses. Interleukins play a major role in gum disease and can be used as diagnostic markers. Objectives. To determine salivary IL-1β and TNF- levels in smokers patients with severe CP. And to assess the association between development of PD and the salivary expression of IL-1β and TNF- .Materials and Methods. Salivary levels of IL-1β and TNF- evaluated using an ELISA assay. Results. In non-smokers, significant differences were observed between CP patients and HC(P=0.062).While in smokers, A significant differences were observed between CP patients and HC (p=0.004).There were significant difference in CRP between the two groups(p=0.027). A Statistically significant difference was observed in gingival health between the two groups, as assessed by CPITN(p=0.006).A statistically significant increase in salivary levels of Interleukin-1β and TNF- was observed in patients with the severe CP than with controls(p<0.001).In patients with severe CP,the correlation between CRP with IL-1β and TNF- were positive with statistically significant (p=0.027, p=0.008),respectively.Similarly a statistically significant positive correlation was observed between smoking with IL -1β and TNF- (p=0.012,p=0.001),respectively. Apositive association observed between pro-inflammatory cytokines identified in this study.Conclusion.Salivary IL-1β and TNF- levels were significantly elevated in severe CP patients compared to healthy controls. These levels increased with the development of the CP and inflammation in smokers patients compared to non-smokers. The association of these interleukins is more common in CP patients who smoke compared to non-smokers.

INTRODUCTION

Periodontitis is an inflammatory disease that affects the tooth supporting tissue and eventually leads to tooth loss. Both oral microbiota and dysbiosis of the host are the main causes of periodontitis (Cheng et al.,2020).
CP damages the supporting structures and periodontal ligament of the teeth,the alveolar bone, the formation of pockets, receding gums, and ultimately tooth loss. It can occur at any age, and is more common in adults(Genco and Williams,2010;Batool et al.,2018).
C-reactive proteins (CRP) is a protein that is synthesized in hepatocytes, under stimulation of IL-6 and IL-1 in the acute phase, and it belongs to the pentraxin family, its levels are elevated in case of chronic inflammation, injury or infection(Tampa et al.,2018).
Cytokines are small proteins that have a major role in modulating immune responses, formerly called monokines or lymphokines depending on the cells that produce them, it either proinflammatory(IL-1,IL-8, TGF-β and TNF-α),or anti-inflammatory (IFN-γ,IL-2,IL-10,and IL-12). The nucleus of any cell has the ability to secrete cytokines,but its main source is macrophages and helper T-cells (Zhang and An,2007).
Among the cytokines,interleukins have a crucial function and are implicated in oral cancer and CP(Tampa et al.,2018). Tumour Necrosis Factor- α (TNF-α) involves nuclear factor kappa B (NF-κB), caspase cascades, transcription factors and activating protein 1(AP-1),and participation in signal regulation, inflammation,cell

growth, and death (Tampa et al.,2018).TNF-α, an inflammatory mediator,has been implicated in carcinogenesis,due to its involvement in chronic inflammatory diseases. Low and sustained production can lead to a tumor phenotype. TNF-α acts as an internal tumor stimulator to block inflammation and carcinogenesis. Based on the trials conducted,it can be used as an indicator of risk,response to treatment,and prognosis for most cancer patients(Ameena and Rathy,2019;Landskron et al.,2014).
Interleukin(IL)-1 is a proinflammatory cytokine that has an effective role as an inflammatory mediator in regulating the immune system, modifying the extracellular matrix and bone, and therefore it is a vital marker of gingivitis diseases(Boch et al.,2001).
Both types of the IL-1 (IL-1α and IL-1β), have similar biological functions, with the exception of interleukin-1beta(IL-1β) being more effective in stimulating bone resorption at CP (Abbasi et al.,2019). IL-1β is pro-inflammatory cytokine,involved in inflammation, immune regulation, and a powerful promoter of bone resorption. The nod-like receptor protein-3 (NLRP3) activates IL-1β, so it is involved in causing gum pathogenesis(Cheng et al.,2020). IL-1β secretion pathway is complex, requiring the occurrence of molecular signals i.e., stress signals or cellular danger, which culminate in molecular platforms and a host of inflammations, which mediate the action of caspase-1 and are essential for the activation of IL-1β(Gomes et al.,2016). All markers are important for

development of specific immune responses. In chronic inflammatory conditions,pro-inflammatory cytokines i.e., IFN-α, IL-1, IL-6,and TNF-y have an important role in bone resorption by activating osteoclasts(Isaza-Guzman et al.,2015; Das et al.,2020).The present study aimed to evaluate levels of some pro-inflammatory cytokines in saliva of smokers with severe chronic periodontitis patients and their relationship with age,BMI,gender and their correlation with each other.

MATERIALS AND METHODS
Study Population
This study was conducted in the clinics of the College of Dentistry,University of Basra,and AL-Sadder Teaching Hospital in Basra Governorate,Iraq.Seventy-six participants their ages ranged from 25---65 years, divided into two groups, the first group included 38 healthy and non-smoking (26 males and 12females) as controls,while the second group included 38 smokers(24 males and 14females)with severe CP enrolled at the Clinical Center at the College of Dentistry at Basra University. From January 2020 to September 2020. Periodontal examination was performed for each donor by a consultant dental surgeon.Also,Clinical evaluation procedures were performed e.g. examination of teeth, periodontal, oral mucosal status,assessment of malocclusion and collection of saliva samples. The body mass index(BMI)of each individual was calculated in a astandard manner. Patients with malignant tumors, infectious diseases, traumatic ulcers and dry mouth, pregnant women, patients with periodontitis other than periodontitis correlated with CP, aggressive periodontitis, periodontitis associated with systemic disease and necrotizing periodontitis were excluded. Also,patients with chronic inflammatory diseases i.e., (psoriasis, arthritis,Sjoegren's syndrome, inflammatory bowel disease) that may affect levels of salivary cytokines. CP patients with at least ten normal teeth, a loss attachment of ≥1 mm in >30% of the sites examined, and a radiographic report of bone loss were included.

Saliva Sample Collection
After examination of clinical periodontal parameter, after informed consent was obtained from each participants and approval of the Ethical Review Committee at AL-Sadder Teaching Hospital. 5 mL of unstimulated whole saliva was collected from each participant in a standard method. Participants were asked to refrain from eating, chewing, and drinking at least one hour before sample collection. Saliva samples were collected between 09:00-11:00AM. Saliva were collected for a 10 min in titration tubes by drooling method. Saliva samples were centrifuged at 10,000×g for 15 min and 4°C, and the supernatant was immediately aliquoted and frozen at −70°C for analysis.

CRPand Cytokine Measurement: Use the Latex Test Kit to perform this assay , CRP was measured by human CRP ELISA kit (Elabscience,Korea). After collecting salivary samples from non-smoking(healthy subjectes), and smokers CP patients, specific cytokines IL-1β,and TNF-α were measured by IL-1β and TNF-α human ELISA kit(Elabscience,Korea) quantitative sandwich immunoassay technique.To assess the effect of periodontal disease on salivary cytokine levels,aCommunity Periodontal Index of Treatment Needs(CPITN)was measured for each patient after saliva collection. CP patients were classified as severe, if their Clinical Attachment Loss(CAL) >5mm. Absorbance was calculated with a Glomax multidetection reader spectrophotometer (Promega, Milan, Italy), for all assays.

Statistical Analysis
SPSS software 20.0 was used for statistically analysis. Data are expressed as mean ±SD.The significance of the difference was assessed for the mean value of the measured parameters between the groups by means of the Student's t-test and chi-square. Correlation was indicated by Pearson correlation tests and $P<0.05$ is considered significant.

RESULT
A non-significant increase was observed in the mean age of severe CP patients compared to HC (p = 0.082). Whereas, the mean BMI and gender between the severe CP and HC group were similar, and were non statistically significant(p=0.485, p=1.000), respectively. Statistically significant difference was observed between patients and HC groups, non-smokers and smokers(P=0.062,p=0.004),respectively.There were asignificant difference in CRP between the two groups(p=0.027). Statistically significant difference was observed in gingival health between the two groups, as assessed by CPITN (p=0.006)[Table 1].Statistically significant increase in salivary levels of Interleukin-1β and TNF-α was observed in patients with severe CP compared with controls(p<0.001) [Table2]. In patients with severe CP, the correlation between CRP with IL-1β and TNF-α were positive with statistically significant(p=0.027,p=0.008), respectively.Similarly a statistically significant positive correlation was observed between smoking with IL -1β and TNF-α (p = 0.012, p= 0.001), respectively.A positive association observed between pro-inflammatory cytokines identified in this study[Table 3].

Table 1. Comparison between chronic periodontitis patients and controls according to age, BMI,Gender, Smoking and CRP.

Variables		CP patients (n=38)	Controls (n=38)	*P-Value*
Age (mean ± SD)		43.36 ± 13.44	25.53 ± 4.61	0.082
BMI (kg/m²)		24.05 ±6.32	23.88 ± 6.48	0.485
Gender				
	Male n(%)	18 (42%)	19 (44%)	1.00
	Female n(%)	26 (58%)	25 (56%)	1.00
Smoking				
	Non smoker	204.80± 86.3	74.51 ± 71.2	0.062
	Smoker	358 ± 64.72	120 ± 17.91	0.004*
CRP(mg/L)		11.82±2.57	4.23±2.26	0.027*
CPITN		7.53±8.14	2.12±2.25	0.006*

* p< 0.05:Differences between patients with chronic periodontitis and healthy group.

Table 2. Salivary Interleukin-1beta, and Tumour Necrosis Factor-α in patients and controls.

Cytokines	CP patients(n=38) (mean ± SD)	controls (n=38) (mean ± SD)	*P-Value*
IL-1β (pg/ml)	528.7 ± 88.1	89.7 ±71.22	<0.001*
TNF- α (pg/mL)	10.34 ±6.85	8.33 ±4.95	<0.001*

* p< 0.05: Differences between patients and healthy group.

Table 3. Correlation between proinflammatory cytokines ,C-reactive protein, and smoking in severe chronic periodontitis patients.

Parameters	TNF-α (pg/ml)	IL-1β(pg/ml)	CRP(mg/l)	Smoking
TNF-α (pg/ml)	-	0.041*	0.008*	0.001*
IL-1β (pg/ml)	0.041*	-	0.027*	0.012*

* p< 0.05: Differences between smokers with severe chronic periodontitis and healthy group.

DISCUSSION

Inflammation is involved in the pathogenesis of numerous chronic diseases. Where there is a relationship between chronic low-grade Inflammation and the development of CP(Tampa et al.,2018).The immune response in CP is unbalanced, resulting in the B-cell subset dominance, macrophages,neutrophils,and T-cell subset,in addition to increased levels of inflammatory interleukins i.e., (IL)-1, IL-6,TNF-α,and interferon(IFN)–γ (Jing et al.,2019;Costantini et al.,2020). A non-significant increase was observed in the mean age of severe CP patients than with healthy subjects (p = 0.082). Whereas, the mean BMI and gender between the severe CP and HC group were similar, and were not statistically significant(p=0.485, p=1.000),respectively [Table 1]. These results are consistent with previous studies(Batool et al.,2018;Gomes et al.,2016), but it differs from the results of the Romanian study, which reported a lower mean age of CP patients than with HC(Monea et al.,2014). Obesity-related inflammation is caused by extra nutrients that initially activate many pathways of metabolic signals, i.e., JNK,NF-kB,and protein kinase pathways (Cheng et al.,2020).Several studies have indicated increase in the prevalence of Periodontal diseases with aging, increased incidence of obesity, diabetes and longer retention of teeth (Gomes et al.,2016;Batool et al.,2018).On the other hand, Gaphor et al.(2014)reported an increase in the prevalence of CP diseases among smokers with the age. This results confirm the validity of our current results. A Statistically significant difference was observed in gingival health between the two groups, as assessed by CPITN(p=0.006) [Table 1].This result is differen from the results of our previous study(Hussein et al.,2020). Smoking is a risk factor, causing gingivitis and increasing the expression of destructive inflammatory cytokines such as IL-1β,TNF-α, and IL-6 in the gingival crevicular fluid(GCF) in smokers compared to non-smokers. Statistically significant differences were observed between patients and HC, non smokers and smokers(P= 0.062,p= 0.004),respectively[Table 1].There were asignificant difference in CRP between the two groups(p=0.027))[Table1]. Studies have reported similar results (Gomes et al.,2016). CRP is an indicator of inflammatory or rheumatic activity, its levels are related in plasma and saliva. Several reports confirm that salivary CRP levels increased during periodontitis,and decreased after successful anti-inflammatory therapy(Tampa et al., 2018). As these studies showed that high levels of CRP in saliva during periodontitis is a marker of the risk of the process for the formation or development of cardiovascular disease. On the other hand, CRP levels increase with disease severity(Ouellet-Morin et al.,2011;Podzimek et al.,2016). There is a correlation between CRP levels and CP prognosis. In the study by Park et al.(2016) an increased CRP/albumin ratio was correlated with prolonged disease and decreased survival rate. These studies support our current findings. Blatt et al.(2018) and Cheng et al.(2020) noted that CRP, ferritin, and hemoglobin can be used as biomarkers in diagnosis and CP progression. The same idea is supported by Tai et al.(2017) in patients with OSCC,which they showed a positive association between high CRP levels and oral cancer, and revealed that CRP level was associated with invasion of localized lymph nodes(Tai et al.,2017).Severe CP can trigger an inflammatory response, triggering the release the cytokines i.e., IL-1β and IL-6(Márton et al.,2019). Periodontal disease(PD) affect concentrations of salivary cytokines (Frodge et al.,2008).However, there were no differences in gum health between study groups. In the current study, a significant increase was observed in salivary levels of IL-1β and TNF-α in patients with CP than with HC(P<0.001)[Table 2].This increase indicates the presence of an inflammatory microenvironment. The results of this study are consistent with other studies(Gomes et al.,2016; Cheng et al.,2020;Das et al.,2020). Immune cell products have an important role in oral mucosa diseases. On the innate response, the molecular phenotypes associated with oral pathogens bind to receptors of host cells, including dendritic cells that activate the inflammatory response through the release of proinflammatory cytokines i.e., IL-1β,TNF-α,IL-6,and IL-17(Das et al.,2020). The altered cytokine response is closely related to the development of CP. Stimulation by inflammatory cytokines suppresses normal cell growth, while oral stimulation of cancer cells with inflammatory cytokines increases regulation of positive cell cycle regulators i.e.,nuclear factor kappa B(NF-κB), activator of transcription(STAT), and signal transducer.The protein kinase pathway is activated by the mitogen/ extracellular signaling(ERK) pathway.Therefore, regulating these factors enhances cell survival and proliferation(Sahibzada et al.,2017). DEATH is a protein bound to TNF-α, encoded by the TRAF2 gene and signaling molecule the RIP, which activates and stimulates the Nuclear Factor Kappa-light-chainenhancer of activated B-cells(NF-κB) pathway, and is involved in cell survival, proliferation, and anti-apoptotic agents. This may explain why TNF-α levels are different in saliva in patients with CP. Barnes et al.(2011) reported that TNF-α is produced mostly by activated macrophages at the site of infection or inflammation, and T-cells. IL-6 production can inhibit TNF-α expression. This finding supports our current results. On the other hand, Li et al.(2003) indicated that the higher regulation of MUC1 expression in oral epithelial cells results from Porphyromonas

gingivalis infection or increases in inflammatory cytokines such as IL-1β, IL-6, and TNF-α. TNF is produced by osteoclasts, and is an important factor that regulates intercellular spacing and is involved in the processes of resorption. The previous study reported significantly higher levels of IL-1β in passive smokers compared to non-smokers(Gomes et al.,2016). On the other hand,Gomes et al.(2016) reported elevated saliva levels of TNF-α and IL-1β in CP groups, and these markers were associated with onset and disease severity, elevation were evidence of disease progression and decreased after treatment. Thus, it can be promising biomarkers in detecting periodontal diseases. However, a previous study divided the concentrations of IL-1β into degrees according to smoking habit, as the concentration of IL-1β was higher in healthy smokers and smokers with gingivitis compared to non-smokers healthy and non-smokers periodontitis who showed an interaction between salivary IL-1β and smoking (Gaphor et al.,2014). Several previous studies have indicated higher IL-1β levels in gingival crevicular fluid (GCF) and saliva cases of CP than with HC(Cheng et al.,2020;Rangbulla et al.,2017). These studies confirm the validity of our current results. Gursoy et al.(2011) noted increased salivary IL-1β in periodontal disease(PD), and its levels are closely related to the development of PD. Therefore, it is considered a biomarker for distinguishing between active and inactive gingival sites.Also,Tâlvan et al.(2017) reported significantly higher levels of the IL-8 and IL-1β in early generalized chronic periodontitis(EGP), rnoderate generalized chronic periodontitis (MGP), and advanced generalized chronic periodontitis (AGP) groups than with HC. While the płasma levels of IL 10 and IL-13were significantly higher at HC compared to the CP groups, this study supports the results of our current study. Therefore, we suggest that hypoxic conditions are essential in increasing IL-1β. Matrix metalloproteinase (MMP)-9 is important indicator of the severity of gingivitis progression, its expression is modulated by IL-1β in the different types of cells involved in periodontitis, including osteoclasts, osteoblasts, cementoblasts, and neutrophils(Du et al.,2019). Belibasakis et al.(2012) indicated that elevated RANKL levels and low levels of OPG are the hallmarks of gingivitis.IL-1β regulates production of the OPG, RANK/RANKL/OPG system, and is related to bone metabolism (Takegami et al.,2017). In fibroblasts, IL-1β increases PGE2 synthesis, which stimulates expression of RANKL. Also, IL-1β increases the expression of CX3CL1 that mediates migration of osteoclast precursors down the osteoblasts layers and osteoblast formation(Matsuura et al.,2017; Cheng et al.,2020). Several studies have reported the negative effect of smoking on the GCF , IL-1β,IL-4,IL-6, and IL-8 levels in HC and early onset periodontitis (EOP) patients (Gaphor et al.,2014; Kamma et al.,2004). Results of the current study indicated higher levels of IL-1β in the saliva of patients who smoking compared to HC, and this could be explained by the fact that smoking causes increase in the expression of the IL-1β in the gingival tissues leading to bone resorption, production of mineral matrix proteins and prostaglandin E2, thus destroying the gum tissue. Elevated levels of IL-1β have been found at the GCF and saliva of tobacco product users. On the other hand, levels of IL-1β are increased when associated with a risk factor such as smokeless tobacco products(STPs) (Javed et al.,2015; Abbasi et al.,2020). Miller et al.(2006) observed that mean levels of IL-1β and Matrix metalloproteinase (MMP)-8 were significantly higher in the saliva of subjects with CP compared to HC. On the other hand, one study reported a strong association between vitamin D and IL-1β in patients with severe aggressive periodontitis(Costantini et al.,2020). Contrary to our results, Teles et al. (2009) indicated that there were non-significant differences in the levels of salivary cytokines (IL-1β,IFN-γ, and TNF-α) between patients with severe CP and HC. These differences could be explained by the difference in saliva collection methods (stimulated or non-stimulated), the storage (time, temperature, addition of protease inhibitors or not), pretreatment (centrifugation time and speed), and the mechanism used to estimate biomarker levels with ELISA versus Luminex.This was confirmed by a previous study(Gaphor et al.,2014). In smokers with severe CP, the relationship between CRP with IL-1β and TNF- α was positive statistically significant (p=0.027, p=0.008), respectively. Similarly,a statistically significant positive association was observed between smoking with IL-1β and TNF- α (p=0.012, p=0.001), respectively. A significant association was observed between both pro-inflammatory cytokines identified in the current study[Table 3]. Results of the current study are agreement with previous studies(Gomes et al.,2016;Batool et al.,2018). Tâlvan et al.(2017) found associations between IL-1B, IL-8, IL-10, and IL-13 in sever CP and between IL-10 and IL-13 in healthy group. Also, they reported an association between plasma concentration of inflammatory interleukins in CP patients, and that an increase in cytokine levels is closely related to the development of periodontal disease,and they noticed a clear relationship between gingivitis and other systemic infections.These studies are in line with our current findings. With this in mind, results of the current study, albeit preliminary, which showed significantly different cytokines levels in the saliva of patients with CP compared to healthy subjects, indicate that saliva, alone or in combination with other molecules, It may be diagnostic markers of severe periodontitis.

CONCLUSION

Asignificantly increased in salivary IL-1β and TNF- α levels at smokers patients with severe CP compared to non-smokers patients and healthy subjects. These cytokines increased with increased inflammation and progression of disease. The use of salivary cytokine-based diagnostics is a biological indicator capable of diagnosing diseases at all stages of CP especially the early stage, and following their development. IL-1β is a potential target for treating gingivitis.

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