Salivary Diagnosis - Clinical Uses in Assessing Oral Inflammation

IULIA IOANA STANESCU[1], ALEXANDRA TOTAN[1]*, FLORENTINA RUS[1], DANIELA MIRICESCU[1], BRANDUSA MOCANU[2], BOGDAN CALENIC[1], MARIA GREABU[1]

[1]University of Medicine and Pharmacy Carol Davila, Faculty of Dental Medicine, Department of Biochemistry, 8 Eroii Sanitari Blvd, 050474, Bucharest, Romania

[2]University of Medicine and Pharmacy Carol Davila, Faculty of Dental Medicine, Department of Periodontology, 19 Calea Plevnei, 010221, Bucharest, Romania

The past decades demonstrated that saliva and its components represent a remarkable diagnosis fluid with valuable clinical uses for both oral and systemic diseases. At the same time it is well established that oxidative stress is involved in a wide number of pathologies, including periodontitis. The specific aim of the present study which included 50 subjects is to determine if saliva can be used in clinical settings to correlate oxidative stress and tissue destruction markers with the severity of periodontal disease. An important oxidative stress marker - 8-hydroxydesoxyguanosine (8-OHdG) and a collagen degradation marker - beta-crosslaps (β-CTX) were quantified in both saliva and gingival crevicular fluid (GCF) using ELISA kits and were found to be significantly increased in the chronic periodontitis group when compared to respective controls ($p<0.05$). At the same time positive correlations were observed between whole saliva and gingival crevicular fluid ($p<0.05$). Significant correlations were also determined between GCF and salivary markers and clinical parameters of periodontal disease. Present results demonstrate that saliva and its components can successfully be used in clinical settings and represents a reliable tool for assessing periodontal disease severity.

Keywords: saliva, periodontal disease, gingival crevicular fluid

Over the past twenty years salivary research has shown that saliva represents a valid alternative diagnostic fluid for multifarious local or systemic diseases, making research in this field a top biomedical priority of the 21st century [1]. A mixture of organic and inorganic molecules, saliva possesses an array of qualities which recommend it as an appropriate diagnosis approach such as effortless collection procedure, facile manipulation and sampling methods, favorable sensitivity, low quantity samples are required for detection, good cooperation with patients during collection, positive correlation between blood markers and the ones found in saliva, the possibility of caring out dynamic studies and most importantly, it is non-invasive [2-4]. One salivary component playing a potential key role in assessing the inflammatory progression locally is gingival crevicular fluid (GCF) [5]. This inflammatory exudate is found in the gingival crevice and can be sampled at the gingival margin. The fluid contains bacterial enzymes, tissue degradation products, mediators of inflammation, extracellular matrix proteins; its biochemical analysis can, therefore, provide unique means of evaluating the initiation and development of periodontal diseases [6, 7].

Periodontitis represents a generally un-reversible inflammatory disease affecting populations of all ages worldwide [8, 9]. The pathology is initiated by specific local microflora residing in the dental plaque as follows: oral bacteria generates an immune reaction in the tooth's surrounding tissues; as a response to this reaction oral epithelia migrates apically, collagen fibers forming the periodontal ligament degrade, alveolar bone is resorbed [10]. Tissue destruction leads to tooth mobility and ultimately to tooth loss.

Numerous studies have shown that an unbalance between antioxidants and reactive oxygen species (ROS) production leads to increased oxidative stress (OS) which is involved in the initiation and development of many systemic and local diseases such as cardiovascular, renal, pulmonary, liver affections, cancers, diabetes or oral conditions [11-14]. Previous studies (including some completed by our group) demonstrate that the initiation and development of periodontal disease are also a consequence of the progressive accumulation of ROS [15]. In this respect, OS markers can be detected in surrounding biological fluids like saliva or gingival crevicular fluid [14, 16].

The present work explored the possibility of including GCF on the short list of fluids that have potential uses as a diagnostic liquid in clinical situations. Thus, our team evaluated the presence or absence in GCF (correlated with salivary levels) of two key markers in the tissue destruction process present in the development of periodontal disease: 8-hydroxydesoxyguanosine (8-OHdG; related to DNA damage) and beta-crosslaps (β-CTX; connected to collagen degradation).

Experimental part

Patient selection

The study was approved by the Ethics Committee of the Faculty of Dental Medicine, University of Medicine and Pharmacy Carol Davila, Bucharest. Informed consent was obtained from each patient and volunteer that participated in the study. The present study included 30 patients with chronic periodontal disease (9 females and 21 males). Twenty healthy subjects with no previous history of periodontal disease and good oral hygiene were used as the control group. The following criteria were used for all participants (chronic periodontitis and control group): no systemic diseases, no history of alcoholism and smoking, no use of antioxidant drugs (such as vitamins) or anti-inflammatory drugs for at least three months prior to the beginning of the study.

* *email: alexandratotan99@gmail.com; Phone: +40722396324*

VARIABLE	CONTROL (N=20)	PATIENTS (N=30)
Age (years)	50.46 ± 5.4	46.21±4.3
Gender (female/male)	10/10	9/21
Smoking status	0	0
Alcohol consumption	0	0

Table 1
DEMOGRAPHIC VARIABLES. DATA SUCH AS AGE, GENDER, SMOKING HABITS AND ALCOHOL INTAKE IS PRESENTED FOR CONTROL GROUP VS. CHRONIC PERIODONTITIS GROUP

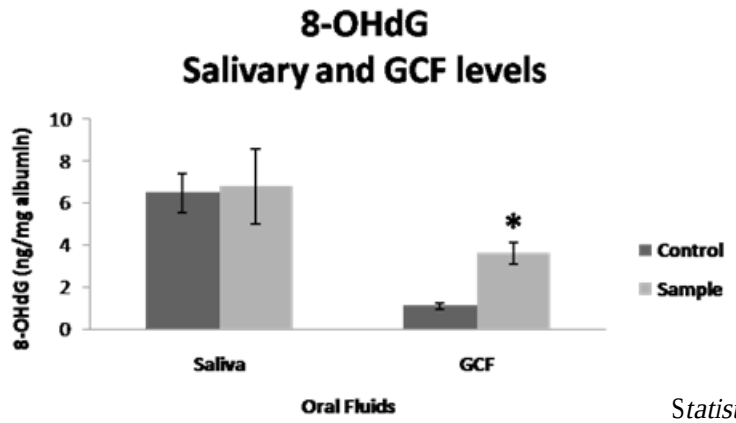

Fig. 1. Salivary and CGF levels of DNA oxidation marker - 8-hydroxy-2-deoxyguanosine. Data is presented as mean values ± standard deviation. Statistical significance was set at p value < 0.05

Clinical parameters

Clinical parameters determined in the study include the plaque index (PI) or the number of dental surfaces with plaque/ total number of examined dental surfaces x100), bone loss higher than 30%, the bleeding index (calculated similarly to the plaque index) and periodontal pockets presence and depth. All examinations were performed by the same examiner.

Saliva and gingival crevicular fluid collection

One day prior to saliva collection the subjects were asked to refrain from drinking and eating. Salivary sampling was performed in the morning between 9 and 10 am in sterile tubes after the subjects rinsed their mouth with 5 ml of distilled water to remove debris and exfoliated cells. Samples were centrifuged, aliquoted and analyzed immediately or frozen until further analysis.

The gingival crevicular fluid was collected on strips of paper inserted in the gingival sulcus of periodontal pockets and maintained for 30 s. Collected strips were included in Eppendorf tubes with 200 µL of phosphate-buffered saline (*p*H of 7.2) and frozen at -20° C.

Oxidative stress and tissue degradation markers detection

Salivary and GCF levels of 8-OHdG and β-CTX were quantified using enzyme-linked immunosorbent assay (ELISA) kits (8-hydroxy-2-deoxyguanosine ELISA kit, Cayman Chemical Company, USA and β-CTX ELISA kit - TSZ Scientific LLC, USA). For both kits, producer's instructions were carefully followed: standards, controls, and samples were pipetted into 96-well plates coated with specific primary antibodies. Following an incubation and a washing step, the substrate solution was added to each well. Color intensity (proportional to the amount of biomarker found in each well) was measured using Stat Fax 303 Plus, Awareness Technology Inc., Palm City, FL, US plate reader.

S*tatistical analysis*

Statistical analysis was performed using Stata IC 11 (Stata Corp.2009. Stata release 11. Statistical Software. College Station TX, USA). Independent sample Student's t-test was used to compare and correlate clinical parameters with biochemical biomarkers. Statistical significance was set at a p-value of <0.05.

Results and discussions

Clinical data

Healthy controls and chronic periodontitis patients were investigated for smoking status, alcohol consumption, plaque index (PI), bleeding index (BI), probing depth of periodontal pockets (PD) (table 1). No statistically significant differences were found for gender and age between the two groups.

Salivary assessment of oxidative stress damage to DNA

Both salivary and GCF levels of 8-OHdG were increased in chronic periodontitis group when compared with respective controls. Thus 8-OHdG in saliva was: 6.47 ± 0.93 ng/mL vs 6.78 ± 1.8 ng/mL while 8-OHdG in GCF was found to be 1.1 ± 0.15 vs. 3.6 vs. 0.51 ng/mL ($p<0.05$, Student t-test) for control vs. periodontitis group respectively (fig. 1). Interestingly, statistical significance could be found only in GCF but not in whole saliva, showing that GCF might be the fluid of choice when assessing DNA damage due to oxidative stress in periodontal disease.

Salivary assessment of bone loss

Whole saliva and GCF levels of β-CTX were significantly elevated in patients with periodontitis vs. control group ($p<0.05$). The results obtained for the two oral fluids and groups are presented in figure 2. Thus in saliva β-CTX was: 0.8 ± 0.09 ng/mL vs. 2.1 ± 0.42 ng/mL ($p<0.05$, Student t-test) while in GCF, β-CTX was found to be: 0.41 ± 0.12 ng/mL vs. 0.77 ± 0.16 ng/mL ($p<0.05$, Student t-test) for control vs. periodontitis group respectively.

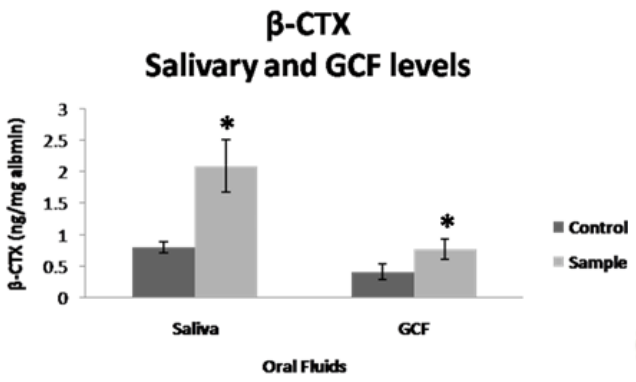

Fig. 2. Salivary and CGF levels of bone loss marker β-CTX (carboxy-terminal cross-linking telopeptide of type I collagen) were assessed in both healthy and periodontitis patients. Data represents mean values± standard deviation. Statistical significance was set at * $p<0.05$.

Periodontitis represents the most widespread chronic inflammatory oral disease, affecting people of all ages worldwide [17]. The main underlying cause of periodontal disease is the presence of supra- and sub-gingival dental plaque together with the associated oral microflora. In most cases, these factors prompt an immune reaction leading to inflammation and affecting the tissues supporting the tooth. Tissue destruction affects the periodontal ligament by the loss of collagen fibers and the alveolar bone by resorption, while the epithelial tissue migrates apically.

Previous research demonstrates that the initiation and development of periodontal disease are not only a result of the immune response due to inflammation, but also a consequence of the gradual accumulation of ROS in saliva, gingival crevicular fluid and tissues that surround the tooth [16, 18]. In all tissues and organs, ROS are found in low concentrations as by-products of cellular metabolism [19]. In normal conditions, ROS have a wide range of physiological roles being involved in cell signaling, cellular division or in the body's defense against bacteria [20]. Main ROS include hydrogen peroxide (H_2O_2), the hydroxyl radical (HO•) and the superoxide anion ($O^{\cdot-}_2$).

Saliva has been proven to be a reliable diagnostic fluid for both oral and systemic diseases (fig.3), accurately reflecting the oxidative stress status in oral tissues. Several studies have explored the possibility to use saliva as a monitoring and diagnosis medium for periodontal disease [21, 22]. However, one drawback is the fact that while total saliva is a very good indicator of health or disease status in the mouth, it cannot give accurate results on individual sites (such as teeth with gingivitis or inflammation) [23]. In this respect, the gingival crevicular fluid has the major advantage of precisely assessing the presence or absence of disease biomarkers around single teeth and therefore, numerous researches have focused on this fluid for the assessment of periodontal disease. [22, 24-26].

Increased concentrations of ROS lead to lipid peroxidation, damage of protein structures and DNA oxidation. 8-OHdG is a nucleoside released in many body fluids such as blood or saliva and represents one of the most reliable markers used in indirectly assessing DNA damage related oxidative stress. Increments in 8-OHdG have also been linked with the initiation of several chronic inflammatory pathologies such as chronic periodontitis. Our results showed that 8-OHdG levels were increased in

Fig. 3. Salivary diagnostic - saliva and its constituents is currently considered a reliable diagnostic fluid. Salivary biomarkers can reflect a wide number of oral and systemic pathologies (oral diseases are shown in blue; general affections are shown in grey)

both total saliva and GCF from patients with periodontitis when compared to normal controls.

Our findings are supported by several previous studies that have focused on the correlation between the levels of 8-OHdG in the oral fluids and the progression of periodontal disease [15,27,28]. Their results also showed higher concentrations of this OS biomarker in both the saliva [15,27,28] and the GCF collected from the inflamed periodontal sites compared to healthy regions of the periodontium [28]. Moreover, similar researches show that salivary 8-OHdG levels vary contingent with the gravity degree of the periodontal disease [29] and that 8-OHdG concentrations are lower after periodontal treatment [30]. Coalescing our results with these findings, it can be concluded that the ROS are involved in the pathogenesis of periodontal disease and that 8-OHdG can be highlighted as an important biomarker for oxidative degradation in periodontal disease.

However, interestingly, no statistical difference could be found between groups in total saliva, while the GCF periodontal group had a statistically higher concentration of 8-OHdG. These results demonstrate that when sampled and analyzed correctly, GCF reflects more accurately the OS status around individual teeth

β-CTX is a biomarker used to evaluate bone resorption, being closely related to collagen degradation. In this respect, in the oral environment, β-CTX can represent a very useful indicator of alveolar bone resorption, a phenomenon diligently linked to the progression and severity of periodontal disease. In a previous study, we focused on the presence or absence of this biomarker in total saliva. Results showed that β-CTX is increased in the whole saliva in patients with periodontal disease vs controls. The present work further extends these findings and investigates β-CTX levels at specific inflammatory sites by sampling CGF from various teeth diagnosed with periodontitis. Data shows that salivary β-CTX levels are higher in periodontitis group than in controls; this result being consistent with our previous findings. At the same time, GCF levels of β-CTX were also markedly increased

with a significant statistical difference between analyzed groups. The outcome of the present work is in concordance with the conclusions of prior studies that also found higher salivary levels of β-CTX in patients with periodontal disease [31]. Other studies investigated the potential use of β -CTX as a biomarker for bone resorption in periodontal disease using serum as the diagnosis fluid and also revealed increased levels for beta-crosslaps compared to healthy subjects [32]. Put together with these findings, our results reflect once again the positive correlation between serum biomarker concentrations and salivary and GCF levels. To the best of our knowledge, the current study is the first to focus on the analysis of β-CTX in GCF as a biomarker for periodontal disease.

While promising as a diagnostic fluid, several limitations exist for GCF analysis: the sample size is small - only a few microliters can be obtained from each site; sampling procedures require a skilled specialist; the selection of teeth or sites from where the fluid should be taken is sometimes difficult.

Conclusions

To sum up, the present work demonstrates that key markers of oxidative stress can be identified and analyzed in saliva and more specifically in its components such as gingival crevicular fluid. In the near future, gingival crevicular fluid may be used in clinical settings as an accurate diagnostic fluid for assessing the relationship between oxidative stress-bone loss and tissue damage in patients with periodontitis.

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