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Possible prognostic biomarkers of periodontitis in saliva

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Abstract. – OBJECTIVE: While both first-line antioxidant enzymes and oxidation products have been considered as markers of periodontal disease, their assessment in the diagnosis of periodontal disease is more complicated. Some, such as superoxide dismutase (SOD, glutathione peroxidase (GPx) and reduced glutathione (GSH), have indicated significant differences between patients with chronic and aggressive periodontitis.

PATIENTS AND METHODS: Participants (101) were divided into a control group of healthy individuals and, following diagnosis, patients with gingivitis, chronic periodontitis, and aggressive periodontitis. Compounds reflecting tissue destruction, inflammatory processes or antioxidant responses, such as sirtuins (SIRT-1, SIRT-2), metalloproteinases (MMP), SOD, GPx, GSH, and glutathione reductase (GR) were measured in saliva.

RESULTS: SIRT-2 levels were significantly increased in all patients. In patients with gingivitis, MMP ($p<0.05$) and GPx ($p<0.01$) were significantly increased. In patients with chronic and aggressive periodontitis, SOD activities were increased ($p<0.001$) while GPx and GR were decreased ($p<0.001$). Relative activities of MMP were higher in patients with aggressive periodontitis.

CONCLUSIONS: Measurements of SIRT-2 and SOD clearly showed increased levels of oxidative stress in cases of periodontitis with a subsequent inhibition of other antioxidant enzymes. Levels of GSH suggest reversibility of the conditions with appropriate intervention. With the assessment of the trends of these selected antioxidant markers, it is possible to determine the prognosis of the disease.

Key Words:
Antioxidant enzymes, Gingivitis, Metalloproteinase, Periodontitis, Sirtuins.

Introduction

Periodontitis is an inflammatory disease affecting approximately 10% of the population. In essence, it is the destruction of periodontal tissues, and supporting tissues of the tooth. Its progression results in the loss of alveolar bone and premature tooth loss[1]. The main cause of periodontitis are microorganisms contained in microbial plaque, with *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythensis* considered to be the main pathogens responsible. Subsequently, external factors play a role, as well as the body's response[2,3]. Thus, the disease is a complex of interactions between pathogenic microorganisms and the host immune response[4,5].

The role of oxidative stress in periodontitis has been studied for decades. The main source of reactive oxygen species is thought to be neutrophils, which are the first line of defense against bacteria. During the process of respiratory inflammation, a superoxide radical is formed. This may then be released into the phagosomal and extracellular space causing the subsequent formation of other radical and non-radical derivatives[4]. As mentioned above, the main cause of periodontitis is the bacteria found in microbial plaque, with the disease's clinical manifestations the result of the inflammatory response of the tissue to this insult. The products of microorganisms trigger the production and release of pro-inflammatory cytokines and various enzymes, whose unregulated action causes tissue destruction. Among these pro-inflammatory groups of compounds there are metalloproteinases (MMPs), which are released from various cells (macrophages, leukocytes, fi-

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broblasts). For normal function of an organism, MMPs are important in the process of extracellular proteolysis, where they are regulated by tissue inhibitors of intercellular matrix metalloproteinases[6]. While cells have mechanisms that prevent oxidative stress under normal circumstances, such as cytoprotective enzymes that scavenge reactive oxygen species[7], the presence of oxidative stress has a negative effect on cells and tissues. Moreover, all mammalian sirtuins appear to be associated with antioxidant and redox signaling pathway regulation[8].

The consequences of oxidative stress, such as lipid peroxidation products and oxidative damage to proteins and DNA, can be used as biomarkers of periodontitis[9-12]. Some studies[13-17] dealt with changes in the activities of antioxidant enzymes in chronic and aggressive periodontitis, and the results showed differences in their activities.

The purpose of this study was to determine the specific activities of these and other selected markers in patients in Eastern Slovakia, although only a lower number of patients could be recruited for the study due to the unfavorable epidemiological situation caused by COVID-19.

Patients and Methods

Patients and Sampling

The Ethics Committee of Louis Pasteur University Hospital approved the study under number 2018/EK/2010. A total 101 patients were enrolled in the study after being informed of the purpose of the study and signing informed consent. Before saliva collection, patients were asked to fast and avoid drinking fluids or brushing their teeth. Collection of saliva took place in the 1st Dental Clinic of the University hospital in the morning between 7:00-9:00 and ran from January 2019 until March 2020. Patients sat upright and spat free-forming saliva for 10 minutes. During collection time saliva samples were stored on ice. After collection, samples were immediately transported to the biochemical laboratory located in the same building. Patients were then divided into 4 groups according to clinical determination. The control group consisted of 43 healthy people. They were without inflammatory changes of the gingiva, without deposits of dental plaque and calculus, without sounding of periodontal pockets or resorption of alveolar bone visible on X-ray. The second group consisted of 17 patients suffering from gingivitis. In the clinical picture, gingival bleeding as a sign of inflammatory changes of the gingiva and possible calculus/plaque deposits were observed. The gingiva was red and edematous soaked. However, the connective epithelium was not disrupted. The periodontal pockets were not probed. Also, no alveolar bone resorption was present on X-ray. In the Community Periodontal Index of Treatment Needs (CPITN) index examination, values of 1 (indicative of any bleeding) and 2 (indicative of calculus deposits) were reported in most sextants[18]. Patients with chronic periodontitis (CP, 23 individuals) formed the third group. During the clinical examination, periodontal pockets were found in the oral cavity. Pocket depth up to 6 mm and more than 6 mm (values of 3 and 4) were measured according to the CPITN and alveolar bone resorption was observed upon X-ray. In addition, bleeding upon stimulus was also observed as a sign of gingival inflammation. Microbial plaque and calculus deposits were present. Patients with aggressive periodontitis (AP, 16 individuals) formed the fourth group. In these predominantly young patients, deep periodontal pockets with a depth of over 6 mm were observed. The gingiva was pale pink with a possible point-like bleeding upon stimulus. Oral hygiene did not respond to widespread damage to the periodontal tissues (with relatively good oral hygiene extensive destruction of periodontal tissues present with history of repeated inflammatory changes of the periodontium). On X-ray, horizontal resorption of the alveolar bone was visible, in some areas vertical and cup shaped. Groups with periodontitis, either chronic or aggressive, represented a generalized form[18].

Antioxidant Measurements

The activities antioxidant enzymes were determined as follows: superoxide dismutase (SOD, EC 1.15.1.1) by the SOD Assay Kit-WST (Fluka, Japan), glutathione peroxidase (GPx, EC 1.19.1.9), and glutathione reductase (GR, EC 1.8.1.7) according to the kit manufacturer's procedures (Sigma-Aldrich, Germany). The reduced glutathione (GSH) concentration was determined by the method described by Floreani et al[19]. Results were normalized to protein content as determined by bicinchoninic acid assay.

Enzyme-Linked Immunosorbent Assay

Enzyme-Linked Immunosorbent Assay (ELISA) was carried out to detect SIRT-1, SIRT-2 and MMP-9 concentrations in saliva of all groups using the SIRT-1 human ELISA kit (Cloud-Clone

Corp., Katy, USA), SIRT-2 human ELISA kit (Abcam, Cambridge, UK) and MMP-9 human ELISA kit (Cloud-Clone Corp., Katy, USA) according to the manufacturer's instructions. Absorption was measured on Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek, Friedrichsthal, Germany) at a wavelength of 450 nm. Standard curves were constructed from the standard solutions included in kits.

Gelatin Zymography

Gelatin zymography was performed for the detection of the MMP-9 isoform on 10 % SDS-PAGE gels copolymerized with 1 mg/mL gelatin. Constant volume of a sample was mixed with loading buffer under non-reducing conditions and loaded into the well. Electrophoresis was run at a constant voltage of 120 V until the loading dye reached the end of gel. Subsequently, the gel was washed 2 x 30 min in the renaturating buffer (2.5 % Triton X-100) with agitation and incubated for 48 h in the incubation buffer (100 mM Tris-HCl pH = 7.4, 10 mM $CaCl_2$, 0.005 mM $ZnCl_2$, 5 mM PMSF). The control gel was incubated under the same conditions in 20 mM EDTA. After incubation, the gels were stained for 1 h with 0.5 % Coomassie Brilliant Blue R-250 in 40 % isopropanol and 10 % acetic acid before being destained for 4 h in 40 % methanol and 10 % acetic acid, and for 24 h in 5 % methanol and 10 % acetic acid. Proteolytic activities were defined as white bands on a blue background and were quantified densitometrically using Image J software. The zymogram of each sample was run in duplicate.

Statistical Analysis

The equality of variances distribution between groups was determined *via* Levene test. Differences between groups were then assessed by Welch's *t*-test and by a nonparametric Kruskal Wallis H-test. Statistically significant results were considered at $p<0.05$.

Results

Due to the epidemiological situation causing a reduction in the number of samples obtained, and the differences in group sizes, it was necessary to perform a Levene's test. The evaluation of the equality of variations between groups has not been confirmed. Consequently, activities of antioxidant enzymes and levels of GSH were expressed in interquartile ranges and medians for each parameter (Table I). As can be seen from the values for SOD, the range is approximately the same in the control group and in patients with gingivitis. It is wider and shifted to higher values in patients with CP and AP. However, by comparison, we found that the mean values of SOD activities in all three groups of patients differed significantly ($p<0.001$) from healthy individuals. In patients with gingivitis, SOD activity was decreased but was increased in patients with CP and AP. By comparing the values in the individual groups with non-parametric one-way ANOVA on ranks, the differences between the groups were significant at $p = 0.039$.

The activities of GPx, in contrast to SOD, showed the opposite trend: in patients with gingivitis, activity was increased compared to control ($p<0.01$) but was significantly decreased in both groups of patients with periodontitis ($p<0.001$). Interquartile ranges were comparable between groups. The narrowest range of values was observed in patients with aggressive periodontitis. Concerning GR, activities in all groups significantly decreased in comparison to control at $p<0.05$ in patients with gingivitis. Although decreases were more pronounced in patients with CP or AP ($p<0.001$). Interquartile range values for GR were also lower, by about half compared to the range found in the control group. However, no differences were found between groups. The observed changes in reduced glutathione levels showed the same trend as SOD activities. Interquartile range values in control and patients with gingivitis were very similar, while a significantly larger range was recorded in patients with CP and AP. GSH levels were, on average, significantly higher in both groups of patients with CP and AP compared to controls ($p<0.001$).

SIRT-1 levels showed no differences from control either in the group of patients with gingivitis nor in either groups of patients with periodontitis (Figure 1). However, increases in SIRT-2 levels were recorded in all three groups of patients with a higher and lower pronounced increase in patients with chronic and aggressive periodontitis, respectively ($p<0.001$, $p<0.05$; Figure 1) when compared to control. Intergroup differences were not significant ($p=0.2794$ and 0.7697). Relative activities of MMP-9 were significantly higher in patients with gingivitis and aggressive periodontitis ($p<0.05$) in comparison to control, with no difference between groups ($p = 0.8406$) (Figure 2).

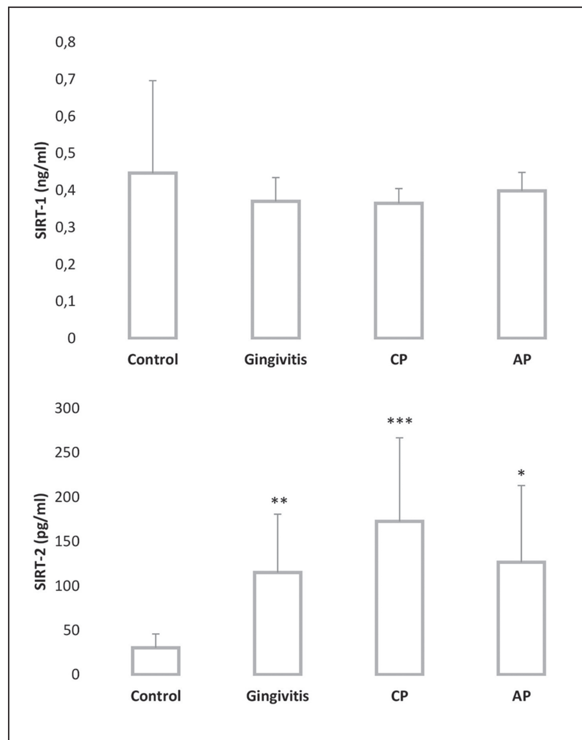

Figure 1. Concentrations of SIRT-1 and SIRT-2 in the saliva of healthy individuals (control) in comparison to patients with gingivitis, chronic (CP) and aggressive periodontitis (AP). Statistical significance at *p<0.05, **p<0.01, ***p<0.001.

Discussion

Periodontitis is an inflammatory disease that develops as the result of the body's immune response to the presence of multiple pathogenic bacteria in the subgingival region. The initial stage, gingivitis, is a reversible condition that, if not treated, leads to periodontitis, the degradation of the fibres of supporting tissues of the teeth[20]. In the progression of inflammatory diseases, the interaction between the production of reactive oxygen species (ROS) by neutrophils at the site of inflammation and the adequate uptake of their excess plays an important role. Apart from the deleterious effects of ROS on cell structures, they play a role in cell signaling. Antioxidant enzymes can also exhibit functions independent of interference with redox activities[21], which can make the interpretation of oxidative stress conditions in periodontitis difficult[12]. At the site of inflammation, the most prevalent and studied ROS are superoxide and hydroxyl radicals, hydrogen peroxide and hypochlorous acid[22].

Superoxide radicals are specifically scavenged by SOD while hydrogen peroxide and substrate peroxides can be converted by catalase and thiol peroxidases. Thiol peroxidases include GPx (and peroxiredoxins) reducing peroxides to water and corresponding alcohols with concomitant oxidation of GSH, which is reduced back to GSH by GR using NADPH reducing equivalents[23]. We have found decreased activities of SOD in patients with gingivitis but increased activities in both chronic and aggressive periodontitis (Table I). Intergroup differences were significant. Similarly, Panjamurthy et al[24] detected increased activities of SOD in patients with periodontitis, and Biju et al[25] in patients after surgical treatment. Furthermore, Duarte et al[26] confirmed the overexpression of SOD2 and GPx1 genes in the gingiva of patients with chronic periodontitis. This corresponds to the findings that induction of SOD is related to the lowering progression of periodontitis[27]. Although other studies[14-16,28] have shown decreased activities of SOD in samples from saliva, sulcular fluid or serum, its activity appears to be of prognostic significance, considering its eminent and irreplaceable role in the first line of superoxide radical scavenging.

Increased expression of SOD (also GPx, GR and GSH synthesis enzymes) is subject to activation of the same regulator[29]. However, the

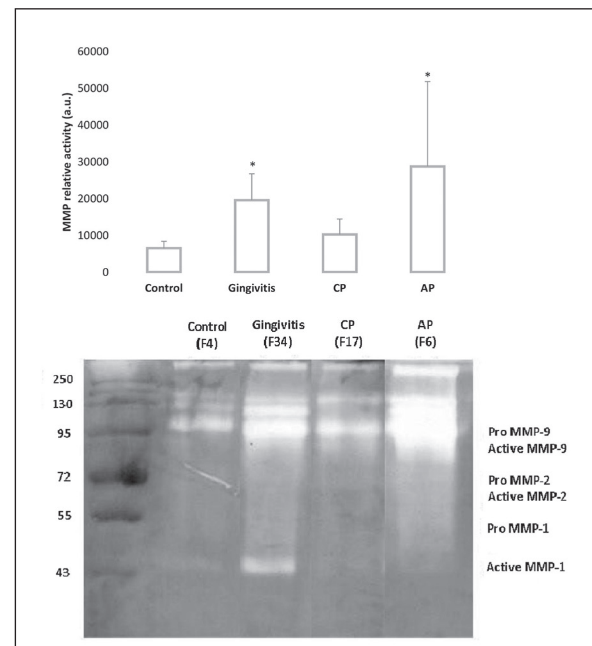

Figure 2. Example of gelatin zymography and expression of relative activity of metalloproteinases (arbitrary unit, a.u.) in the saliva of healthy individuals (control) in comparison to patients with gingivitis, chronic (CP) and aggressive periodontitis (AP). Statistical significance at *p<0.05.

Table I. Characteristics of enzymatic and non-enzymatic antioxidants described as interquartile range values and median in healthy patients (control), patients with gingivitis, chronic parodontitis (CP) and agressive parodontitis (AP).

Group/parameter	SOD (Q1-Q3) med (μkat/mg prot)	GPx (Q1-Q3) med (μkat/mg prot)	GR (Q1-Q3) med (μkat/mg prot)	GSH (Q1-Q3) med (nmol SH/mg prot)
Control	(0.057-0.092) 0.067	(0.041-6.809) 2.917	(0.061-6.118) 1.098	(0.053-0.539) 0.285
Gingivitis	(0.041-0.075) 0.049***	(0.028-7.194) 4.111**	(0.083-3.611) 0.778*	(0.051-0.455) 0.224
CP	(0.105-0.201) 0.139***	(0.033-5.342) 1.203***	(0.022-2.759) 0.695***	(0.071-2.058) 0.289***
AP	(0.095-0.221) 0.109***	(0.037-4.049) 2.025***	(0.037-3.346) 0.698***	(0.035-0.873) 0.446***
p	0.039	0.281	0.973	0.736

Statistical signifikance of *t*-test at $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

concomitantly detected activities of synergically operating GPx and GR must not have been as high as they were found to be in e.g., Dahiya et al[13] or Sreeram et al[17]. In our study of patients with both chronic and aggressive forms of periodontitis, GPx activities were significantly lower than controls. Similarly, low GPx activities were also found in the study by Borges et al[30] and Miricescu et al[31]. A simple reason for this phenomenon is the overproduction of peroxides due to the increased activity of SOD, which leads to substrate inactivation of GPx, even at millimolar concentrations[32]. Increased conditions of oxidative stress are then confirmed by significantly lower GR activities. GR inactivation occurs due to an increase in the concentration of lipid peroxidation end products[33].

Thus, it appears that the concentration of GSH is a decisive indicator for the assessment of disease prognosis. GSH is not only a substrate in the GPx-catalysed reaction, where its efficient back-reduction through GR ensures optimal catalysis. It can itself demonstrate the ability to scavenge superoxide, hydroxyl, and lipid peroxyl radicals, and hydrogen peroxide[34]. It is not, therefore, uncommon to expect reduced GSH levels, like the study by Gumus et al[35] or in inflammation at all[36]. We found significantly higher GSH levels in patients with both chronic and aggressive forms of periodontitis when compared to healthy individuals (Table I). Our results are not isolated as elevated levels were also noted by Borges et al[30], de Araújo et al[37], Kırzıoğlu et al[38] and Rai et al[39], together with lower levels with gingivitis[40]. It is therefore not clear if efficient recycling of GSH is a reason to maintain levels, but rather its *de novo* synthesis. Lower levels of GSH in the group with gingivitis likely reflect early progression to a more severe stage of the disease.

SIRT-1 and SIRT-2 are NAD^+-dependent deacetylases that often exhibit antagonistic effects in diseases where inflammation and oxidative stress occur. SIRT-1 plays a crucial role in gene expression, metabolism, stress resistance, and apoptotic cell death. It has been previously demonstrated the anti-inflammatory effects of SIRT-1. In periodontitis, SIRT-1 has a protective role. The reduction of SIRT-1 levels leads to the activation of JNK/NF-κB pathways and release of pro-inflammatory cytokines and MMP-9 in periodontal fibroblasts[41]. In addition, allosteric activation of SIRT-1 was shown to enhance the Nrf-2 antioxidant pathway in gingival tissue[42]. On the other hand, SIRT-2 upregulation was seen in inflamed gingival fibroblasts[43]. We, in turn, detected an increase in SIRT-2 in gingivitis, chronic and aggressive periodontitis; SIRT-1 did not differ significantly from control, as was detected in the study of Caribé et al[44]. This could be caused by cellular localization, as SIRT-2 is in the cytosol. SIRT-1 shuttles between the cytosol and nucleus and does not necessarily leak out when cellular permeability is disrupted. The mechanisms by which SIRT-1 and SIRT-2 come to be found in saliva have yet to be fully explained. These findings not only insinuate but confirm that oral fluids may represent a significant source of discriminatory biomarkers for local, systemic, and infectious disorders[45].

Finally, matrix metalloproteinases (MMPs) are a major group of enzymes responsible for the degradation of the extracellular matrix. Destruc-

tion of periodontal tissues during periodontitis is caused by collagenases, which are a subset of matrix metalloproteinases. Under physiological conditions, tissues are protected by tissue metalloproteinase inhibitors (TIMPs). MMPs are zinc-dependent endopeptidases obtained predominantly from polymorphonuclear cells during the acute stages of periodontal disease[46]. For the assessment of gingival ECM (extracellular matrix) damage, we further analyzed total salivary gelatinolytic MMPs. These were significantly augmented in the saliva of patients, except in chronic cases, suggesting a difference between the chronic and aggressive forms. More specifically, the MMP-9 isoform showed an increasing trend as also found by Rai et al[46] in the saliva of patients. Other metalloproteinases also contribute significantly to inflammation and the destruction of the periodontium.

Conclusions

We monitored changes in several substances involved in influencing and regulating stress conditions, inflammatory processes, and tissue destruction, such as SIRT-1, SIRT-2, and MMP in addition to the first line defense antioxidant markers SOD, GPx, GR, and GSH. The results from saliva indicated altered, oxidative stress conditions and degradation processes. The number of individuals involved in the study is no different from others published, the numbers of which are mentioned in more detail in Bains and Bains[47]. As the monitored parameters show variability between individuals, we can say that selected parameters can be used, not in the diagnosis, but in the prognosis of the disease. While superoxide dismutase activity can be considered as the first decisive data point, the prognosis can be determined more precisely from the interplay between other first-line antioxidant markers.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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