Original Research Article

Hydrogen peroxide in patients with periodontal disease – pilot study

ABSTRACT

Purpose: To assess the levels of hydrogen peroxide (H_2O_2) in crevicular fluid samples collected from patients with periodontal disease (PD) and periodontally healthy individuals.

Methods: Crevicular fluid samples were collected from three diseased sites in 20 PD patients and from three healthy sites in 11 periodontally healthy patients (control). Plaque indices, probing depths, clinical attachment levels, and bleeding on probing were measured.Subsequently, the collected samples were categorized into three groups: a gingivitis group (n=8), a periodontitis group (n=12), and a control group (n=11). The quantification of H_2O_2 content within these samples was analyzed using an H_2O_2/peroxidase test kit.

Results: Higher levels of H_2O_2 were found in the periodontitis group compared to group control and gingivitis ($p<0.0001$). Furthermore, the gingivitis group also shown elevated H_2O_2 levels compared to the control group ($p<0.0001$).

Conclusions: The findings suggest that the severity of periodontal disease could be associated with elevated levels of H_2O_2 in gingival crevicular fluid. Hydrogen peroxide may potentially serve as a biomarker for disease progression or for products aimed at enhancing H_2O_2 release in periodontal treatment. However, further investigations with larger sample sizes and longitudinal designs are necessary to corroborate and expand upon these preliminary observations.

Keywords:Periodontal Diseases; Periodontitis; Hydrogen Peroxide; Oxidative Stress.

1. INTRODUCTION

The development of oral diseases, such as periodontal disease (PD) and dental caries, is consistently linked to pathogenic bacterial biofilm formation [1]. This transition from non-pathogenic to pathogenic biofilm involves intricate interactions among various microbial and host factors. A distinctive feature of oral biofilm is its dense and diverse composition of microbial species [2] facilitating intercellular contact and interactions within and between species [3–5].

The progression of PD begins with interactions among oral microorganisms in the development of biofilm, from the early stages of colonization to form the mature supragingival and subgingival biofilms [6,7]. These interactions occur at different levels, including physical contact, metabolic exchange, communication mediated by small signaling

molecules, and genetic material transfer [8]. A notable feature of oral bacteria is their capacity for coaggregation which is defined as specific cell-to-cell recognition occurring between genetically distinct cell types [2]. Therefore, bacterial interactions play an important role in the PD progression which a gradual shift from low initial bacterial diversity in supragingival biofilm to high diversity in subgingival biofilm [8,9].

Biofilm formation commences with the adherence of primary colonizers, situated in the outer layers of the biofilm, influenced by diverse environmental, host, and bacterial conditions [2]. These initial colonizers bind to oral surfaces, serving as substrates for subsequent colonization by potential periodontal pathogens [10,11]. Evolving interaction mechanisms foster microbial imbalance, leading to an increase in pathobionts and a decrease in commensal bacteria, resulting in dysbiosis [12,13].

In the context of pathobiont proliferation, the biofilm undergoes modulations that encompass heightened extracellular matrix deposition, thereby constricting nutrient and oxygen availability in the inner strata [1]. Remarkably, specific commensal species within the biofilm harness oxygen (O2) to synthesize hydrogen peroxide (H2O2), engendering a conducive milieu for the expansion of anaerobic microorganisms.

Consequently, this consortium of microorganisms elicits localized inflammation, inciting immune system engagement via the orchestration of inflammatory mediators. Noteworthy among these orchestrations are peroxidases, conventionally recognized as host-protective entities, which orchestrate the production of antibacterial agents, including lactoperoxidase (LPO) and myeloperoxidase (MPO) [12].

The dysbiosis induced in the host through the inflammatory process, coupled with an elevation in the quantity of pathogenic microorganisms, can lead to an escalation in reactive oxygen species (ROS), which exacerbate periodontal disease [14]. In reaction to the formation of ROS, there is an augmentation in the synthesis of antioxidants, encompassing LPO, which holds responsibility for the degradation of H2O2 [12]. Notably, H2O2 is generated by both oral microorganisms and the intricate cellular and enzymatic systems of the host [15].

To assess the influence of H2O2 in different clinical presentations of PD, this study evaluated the amount of H2O2 present in the gingival crevicular fluid collected from patients with PD and periodontally healthy individuals.

2. MATERIAL AND METHODS

The participants were informed about the purpose and methodology of the study and signed a consent form that had been previously approved by the Ethics Committee (CAAE24833919.9.0000.0081). A total of 20 patients with PD and 18 control patients were included in this study for the collection of gingival crevicular fluid. A minimum of 3 diseased sites from patients with periodontal disease and 3 healthy sites from control patients were selected. These samples were subsequently collected and stored at a temperature of -80°C. The assessment involved quantifying the amount of H_2O_2 present in both the diseased and healthy sites.

Clinical periodontal evaluation, including Plaque Index (PI), Probing Depth (PD), Clinical Attachment Level (CAL), and Bleeding on Probing (BOP). Clinical examinations were conducted using a Williams probe, identifying 3 diseased sites with probing depths between 4 and 6 mm and clinical attachment loss between 3 and 4 mm. Additionally, healthy sites were selected with probing depths equal to or less than 3 mm and without clinical attachment loss, across distinct teeth. Before fluid collection, supragingival biofilm was

gently removed using sterile cotton, the area was isolated with cotton rolls, and meticulously dried with gentle air streams.

Based on the outcomes, the participants were categorized into 3 groups [16,17]: a control group comprising 11 patients, a gingivitis group with 8 patients, and a periodontitis group with 12 patients.

2.1 Gingival fluid samples

All patients underwent a clinical examination to collect data on probing depth (PD), clinical attachment level (CAL), and plaque index (PI). The samples were collected from six randomly chosen teeth in each patient, comprising three diseased sites and three healthy sites. Gingival fluid was collected using a paper strip (Periopaper – ProFlow Inc., Amityville, NY, USA) for 30 seconds. The volume of fluid was quantified (Periotron 8000 – ProFlow Inc., Amityville, NY, USA), and the sample was stored at -80 °C until analysis.

2.2 Quantification of Hydrogen Peroxide

The concentrations of H_2O_2 in gingival fluid samples were measured using an H_2O_2/peroxidase assay kit (Amplex Red H_2O_2/Peroxidase assay kit; Molecular Probes, Invitrogen). Fifty µl of diluted gingival fluid samples were incubated with 50 µl of a solution containing 0.05 M NaH2PO4 (pH 7.4), 0.2 U/ml horseradish peroxidase (HRP), and 25.7 mg/ml Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) for 2 hours at 37 °C. Following incubation, the absorbance of the reaction mixture was read at 560 nm. Absorbance readings were compared with those obtained from a standard curve of H_2O_2 (0-40 µM). Results are expressed as H_2O_2 concentrations in µM.

2.3 Statistical Analysis

The data were analyzed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). After assessing normality and homoscedasticity using the Shapiro-Wilk and Levene tests, respectively, non-normally distributed data were subjected to Kruskal-Wallis and Mann-Whitney tests. The significance level for all tests was set at 5%.

3. RESULTS AND DISCUSSION

The probing depth data show a statistical difference (P< .0001) among all groups (Table 1). Analyzing the CAL, we observed that the periodontitis group exhibited a clinical attachment loss of 6.06 mm, with a statistically significant difference (P<0.0001) compared to the gingivitis and control groups, both showing 0 mm of attachment loss (Table 1).

The PI and GI clinical data showed the same results (score 1), with a statistically significant difference compared to the control group (score 0) (Table 1).

H_2O_2 levels were higher in the periodontitis group (11.19 µM) compared to the other groups, with the gingivitis group measuring 7.76 µM and the control group measuring 5.55 µM. There was a statistically significant difference (P< .0001) among all groups (Table 1).

Table 1. Clinical parameters and levels of H_2O_2 for each group.

Parameter	Control	Gingivitis	Periodontitis	*P*-value
PI score	0.40 (±0.91) [b)]	1.21 (±0.58) [a)]	1.35 (±0.49) [a)]	*P*<.0001
GI score	0.32 (±0.90) [b)]	1.71 (±0.82) [a)]	1.82 (±0.88) [a)]	*P*<.0001

PD (mm)	0.76 (±0.44) [c)]	2.14 (±1.02) [b)]	5.47 (±1.77) [a)]	*P*<.0001
CAL (mm)	0.12 (±0.33) [b)]	0.14 (±0.36) [b)]	6.06 (±2.46) [a)]	*P*<.0001
H_2O_2 (µM)	5.55 (±0.56) [c)]	7.76 (±0.93) [b)]	11.19 (±1.57) [a)]	*P*<.0001

PI score: plaque index score, GI score: gingival index score, PD: probing depth, CAL: clinical attachment level, H_2O_2: quantification of H_2O_2. Lower case letters in superscripts represent the comparison between the columns according to the line. Different letters show statistical difference.

Commensal species of the biofilm suppress pathobionts through the production of H_2O_2[12], ensuring a survival advantage in this microbiome [18,19]. However, catalase and peroxidases, at high concentrations, can neutralize this effect and contribute to dysbiosis, allowing the growth of pathobionts [12]. The increased presence of pathogenic microorganisms and the host's inflammatory response led to an increase by products such as H_2O_2, which exacerbates PD due to the generated oxidative stress [20].

The presence of pathogenic bacteria in the periodontal tissue primarily results in the activation of phagocytes (neutrophils and macrophages) as antibacterial agents [21]. There is also evidence linking ROS to periodontal destruction, with the presence of neutrophil infiltration being the main event in the host's response to bacterial aggression [22,23]. Several endotoxins, such as proteases and lipopolysaccharides, produced by bacteria cause inflammation and PD progression. Myeloid cells, including macrophages, have a tolerance mechanism to these toxins, suppressing inflammation and promoting tissue regeneration. However, oxidative stress inhibits the host cells' tolerance mechanism to endotoxins released by pathogenic bacteria, thereby aggravating PD from the host's side [24]. Furthermore, oxidative stress caused by H_2O_2 induces premature aging of periodontal ligament cells [25].

Given the need for further studies that enhance the understanding of this process, investigations regarding the influence of H_2O_2 become relevant in this research, considering the collection of crevicular fluid in patients with PD (gingivitis and periodontitis) and periodontally healthy patients [26]. Our results suggest that the concentration of H_2O_2 increased according to the PD progression in the analyzed patients, indicating the influence of H_2O_2 on the worsening of PD.

Regarding the collection method, it is important to note that saliva also contains components that can be used to monitor systemic status and oral health, as it represents a combined sample from all periodontally affected sites, providing an overall assessment of disease status [27]. However, since PD is a site-specific pathological condition, the collection and analysis of crevicular fluid from periodontal pockets are more accurate for diagnosis [5,28]. Therefore, the use of crevicular fluid samples reflects periodontal conditions by containing host cells, microorganisms from the dental biofilm, as well as their cellular products, tissue serum concentrations, and inflammatory mediators [29]. Thus, investigating the level of H_2O_2 in crevicular fluid is important as certain microorganisms generate H_2O_2 in their metabolic processes, which can be used as a biomarker for PD diagnosis [12,14,28]

4. CONCLUSION

The quantification of H_2O_2 levels in the studied sample indicates a higher quantity in patients with periodontitis, suggesting a biological imbalance that may affect periodontal tissues.As this is a pilot study, a larger sample size is required to substantiate the variation in H_2O_2 levels corresponding to the severity of PD clinical presentations. The elevated H_2O_2 concentration observed in periodontitis patients in this study provides a strong foundation for future investigations exploring the utilization of products aimed at enhancing H_2O_2 release in periodontal treatment. H_2O_2 is acknowledged for its antimicrobial properties and potential to suppress pathobionts within dental biofilms, thereby conferring a survival advantage to commensal species in the biofilm. Nevertheless, further comprehensive research and clinical trials are essential to validate this approach and ascertain the optimal H_2O_2 concentrations

for safe and effective treatment.

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