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ORIGINAL ARTICLE

Comparative estimation of sulfiredoxin levels between chronic periodontitis and healthy patients – A case-control study

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Abstract

Background: Growing evidence suggests that oxidative stress forms a key component in the etiopathogenesis of periodontitis. Studies have shown potential antioxidants responsible for combating the pro-oxidants which stress the periodontium. But, peroxiredoxin-sulfiredoxin system is the least explored in periodontal disease.

Methods: A case-control study was conducted on 30 participants who fulfilled the inclusion criteria from the Department of Periodontics, Saveetha Dental College and Hospital, Chennai, India. The patients were divided into two groups: 1) Group A- healthy controls (n = 18), 2) Group B- patients with generalized chronic periodontitis (n = 17). Following clinical examination, gingival tissue samples were procured from both the groups and subjected to protein quantification by Lowry method. The samples with adequate protein concentration (n = 30) from the two groups were further analyzed by enzyme-linked immunosorbent assay for estimation of sulfiredoxin levels.

Results: Sulfiredoxin levels were significantly higher in the gingival tissues of chronic periodontitis patients (171.20 ± 16.97 ng/mL) than in healthy controls (131.20 ± 22.87) with $P < 0.001$. Also, the levels of sulfiredoxin in gingival tissue of periodontitis patients positively correlated with site-specific probing depth (r = 0.67; $P = 0.007$) and clinical attachment level (r = 0.55; $P = 0.035$).

Conclusions: The present study was a novel attempt to estimate the levels of sulfiredoxin which was significantly elevated in the diseased sites of patients with chronic periodontitis. Future studies are required to probe the role of sulfiredoxin in the etiopathogenesis of periodontal disease.

KEYWORDS
antioxidants, oxidative stress, periodontitis, reactive oxygen species

1 | INTRODUCTION

Periodontitis is a chronic inflammatory disease mediated by host-bacterial interactions and manifested by damage to the periodontal tissues which may progress to tooth loss.[1] There is a dysregulation of the host immune response to the bacterial plaque that causes an exaggerated inflammatory response and results in periodontal tissue destruction.[2,3] The neutrophils are the principal immune cells present in the junctional and crevicular epithelium and through their phagocytic action, they produce very high levels of reactive oxygen species (ROS) to mediate the killing of microorganisms.[4] Various studies have documented that excessive ROS production from hyperactive neutrophils in periodontitis could result in tissue destruction due to the oxidation of DNA, protein or lipid in the cells.[5,6] Gingival and periodontal ligament

fibroblasts, which form the majority cell population in the periodontium have also been observed to generate ROS when stimulated.[7,8]

Despite the protection offered by the inflammatory response to the periodontium, prolonged exaggerated responses could result in the activation of redox-sensitive transcription factors such as activator protein-1 (AP-1) and nuclear factor kappa beta (NF-kß). It was hypothesized that the endogenous ROS from hyper-responsive neutrophils or fibroblasts within the periodontal structures might cause the activation of AP-1 and NF-kß with the resultant deleterious proinflammatory and tissue-damaging effects observed in periodontitis. However, it has been ably supported in the literature that such events could occur due to oxidative stress and it might be inhibited by thiol compounds.[9–11] Numerous studies reported that ROS generated by periodontal inflammation could gradually diffuse into the bloodstream and affect other organs.[12,13] Hydrogen peroxide is one among the noxious ROS being released during these events and it is responsible for cellular apoptosis and tissue damage.[14]

The enzymatic antioxidants in action against hydrogen peroxide, namely, catalase (CAT), peroxiredoxin (Prx), thioredoxin (Trx), glutathione peroxidase (Gpx) have been grouped as 'peroxidases'. The scavenging of hydrogen peroxide is largely mediated by the peroxiredoxin-sulfiredoxin (Srx) system of antioxidants.[15] The local rise in ROS is responsible for the inactivation of Prx which enables hydrogen peroxide to act as an intracellular cell signaling agent. The Srx enzyme is solely responsible for Prx activation by the reduction of the cysteine sulfinic acid of the Prx to its stable thiol state.[16] The Srx enzyme exerts its action on Prx to efficiently remove the hydrogen peroxide moieties and attenuate intracellular oxidative stress.[17]

A study done by Duarte et al in 2012 showed that the expression profile of Prx 1 and 2 was found to be upregulated in the gingival biopsies of patients with type 2 diabetes and periodontitis.[18] There is controversy regarding the expression of antioxidants and the periodontal status. However, few authors reported contradicting evidence that periodontitis was related to a decreased expression of antioxidant defenses,[19,20] whilst others suggested that the antioxidant defenses were upregulated in periodontal disease.[21,22]

Expression of sulfiredoxin was observed in macrophages, neurons, hepatocytes, alveolar cells, and in tumor cells.[23–26] It has been implicated in a variety of oxidative stress-induced conditions such as atherosclerosis, chronic obstructive pulmonary disease, and in a wide range of carcinomas involving the various organs of the body.[27–30] A direct link between sulfiredoxin and periodontal disease has not yet been elucidated. To the best of the authors' knowledge, the present study is first of its kind to estimate and compare the levels of sulfiredoxin in patients with chronic periodontitis (CP) and healthy controls.

2 | MATERIALS AND METHODS

The study population consisted of 35 participants (20 males and 15 females) aged 20 to 60 years, attending the outpatient section of Department of Periodontics, Saveetha Dental College and Hospital, Chennai, India. The study was conducted from December 2014 to May 2015. The study groups were as follows: Group A, healthy controls (n = 18); group B, patients with generalized CP (n = 17). The diagnosis of CP was made in accordance with the American Academy of Periodontology International Workshop for Classification of Periodontal Diseases, 1999.[31] Written informed consent was obtained from all the study participants. Ethical clearance was obtained from the Institutional Ethics Committee and Review Board (IHEC/SDMDS13PER3).

Based on the consensus classification, the participants were categorized as generalized CP when 30% of the sites were affected by the disease. Also, probing depth (PD) ≥5 mm and clinical attachment level (CAL) ≥4 mm in at least 10% of the sites were mandates, for group B participants. The patients indicated for crown lengthening procedure with PD ≤3 mm, no loss of CAL and absence of gingival inflammation (gingival index [GI] = 0) were deemed as healthy controls. The following patients were excluded: 1) Smokers, 2) history of any systemic diseases that can alter the course of the periodontal disease, 3) history of periodontal therapy in the preceding 1 year, 4) vitamins A/C/E or any form of antioxidant therapy within the last 3 months, 5) use of antibiotics, 6) pregnant/lactating women. The selected patients were subjected to full-mouth periodontal probing and charting. The parameters assessed were both, full-mouth and site-specific probing depth (F-PD, S-PD), clinical attachment level (F-CAL, S-CAL), gingival index (F-GI, S-GI) and plaque index (F-PI, S-PI).[32,33] Both PD and CAL were measured using a periodontal probe.* The clinical examination and the site selection for procurement of samples were performed by a single trained and calibrated examiner (AR). The site with the persistently deep and active (presence of clinical signs of inflammation, F-GI, S-GI ≥ 1) periodontal pocket following scaling and root planing was selected for sample procurement in group B participants. The tissue samples were collected at the time of flap surgery (modified Widman flap) by trimming the inner lining of the flap. The gingival tissue from group A individuals was procured during crown lengthening procedure. The tissue samples from both the groups were washed in saline and stored in 0.5 mL of protease inhibitor solution.† It was transported on dry ice and stored at −80°C until assayed.

* Williams Periodontal Probe, Hu-Friedy, Chicago, IL

† P8340 Protease inhibitor cocktail, Sigma-Aldrich, St. Louis, MO

2.1 | Preparation of tissue homogenate

The gingival tissue samples were dissected into small pieces on the ice to prevent degradation by proteases. It was done using scissors and solubilized in 100 μL of protease inhibitor solution* and 200 μL of radioimmunoprecipitation assay buffer. For ≈5 mg piece of tissue, 300 μL of lysis buffer was added, rapidly homogenized using mortar and pestle and transferred into 1.5 mL plastic tubes.† Centrifugation was done for 20 minutes at 15,000 rpm at 4°C in a microcentrifuge, and the plastic tubes were placed on dry ice. The supernatant was aspirated into a fresh tube.

2.2 | Protein estimation using Lowry method

The protein quantification of all the samples was performed using the Lowry method.[34] The blanks were prepared by pipetting 200 μL of milliQ water (mQ H_2O), 100 μL reagent A (alkaline copper tartrate solution) and 900 μL of reagent B (Folin reagent). The sample solution was prepared by mixing 195 μL mQ H_2O, 5 μL of the sample, 100 μL of reagent A and 900 μL of reagent B. The color change was evaluated using a spectrophotometer at 750 nm for each sample and recorded. A total of five samples (group A, n = 3; group B, n = 2) could not be assayed further because of poor protein concentration.

2.3 | Assay procedure

The samples were thawed at room temperature for 30 minutes before the assay. Commercially available enzyme-linked immunosorbent assay (ELISA) kit‡ for human sulfiredoxin was used in the study. The quantitative sandwich ELISA utilized a purified human Srx coated microelisa strip plate. The protein concentration was fixed as 100 μg, and the volume of each sample was procured appropriately. Each sample was then brought to a final volume of 100 μL by the addition of the assay diluent provided in the kit. All the standards (100 μL) and samples were run in duplicate. The enzyme-linked conjugate specific for sulfiredoxin was added and allowed to incubate for 60 minutes at 37°C. The plate was washed manually to remove any unbound conjugate, following which the chromogen solutions were added to the wells. The color was developed in proportion to the amount of sulfiredoxin bound. The color reaction was stopped, and the intensity of the color was measured using ELISA reader using 450 nm as the primary wavelength.

The minimum detectable dose of Srx ranged from 3.12 ng/mL to 100 ng/mL and the sensitivity was 1.0 ng/mL. Both intra-assay and interassay precision were <15%. The specificity showed that there was no significant cross-reactivity between human Srx and analogues.

2.4 | Statistical analysis

The obtained results were subjected to normality tests such as Kolmogrov-Smirnov and Shapiro-Wilk tests. The resultant data showed that they followed a parametric distribution. Independent t-test was performed to compare the mean sulfiredoxin levels between the two groups. Pearson correlation test was done to assess the correlation of full-mouth and site-specific clinical parameters with the sulfiredoxin levels. Multiple linear regression analysis was performed for variables (S-PI, S-GI, S-CAL, S-PD) associated with sulfiredoxin in chronic periodontitis. All analyses were conducted using statistical software.§ $P < 0.05$ was considered to be statistically significant.

3 | RESULTS

The study included 35 participants, of which 30 tissue samples which had sufficient protein concentration were subjected to ELISA. The descriptive statistics of the clinical parameters from both the study groups have been depicted in (Table 1). The S-PD for patients with chronic periodontitis ranged between 5 to 10 mm with the mean value of 6.44 ± 1.94 and an S-CAL average of 7.97 ± 2.65. The results of the present study showed that mean sulfiredoxin levels were higher in the tissue samples of group B patients (171.20 ± 16.97 ng/mL) than in group A patients (131.20 ± 22.88 ng/mL). The difference between the two groups was statistically significant (t-value of 5.44, $P < 0.001$) (Table 2) (Figure 1).

In patients with chronic periodontitis, significant positive correlation of sulfiredoxin levels was found with S-PD (r = 0.67; $P = 0.007$) and S-CAL (r = 0.55; $P = 0.035$). On the other hand, parameters such as plaque index and gingival index (F-PI, S-PI, F-GI, S-GI) did not have a significant correlation with the levels of sulfiredoxin. Among healthy controls, the F-PI, F-GI, and F-PD showed a mild negative correlation with the sulfiredoxin levels, but it was not statistically significant (Table 3).

The multiple linear regression analysis (MLR) of site-specific clinical parameters in patients with chronic periodontitis (n = 15) revealed that there was no statistically significant predictor of sulfiredoxin levels. However, S-PD had borderline significance with a regression coefficient value (ß coefficient = 9.32) and a P value of 0.06 (Table 4). Also, it can be noted that there is a statistically significant difference in the mean age among the two groups (Table 1). To assess the confounding effect of age, the MLR analysis along with

* P8340 Protease inhibitor cocktail, Sigma-Aldrich, St. Louis, MO

† Eppendorf tubes, Eppendorf, Hamburg, Germany

‡ Catalog no. MBS9341972, MyBiosource, San Diego, CA

§ SPSS v.17 software, IBM, Armonk, NY

TABLE 1 Characteristics of the study population

Characteristics	Healthy n = 15	Chronic Periodontitis n = 15	*P*-value
Age	25	40.46	0.000
Sex (Male:Female)	9:6	9:6	
Plaque index – Full mouth (F-PI)	0.18 ± 0.17	1.41 ± 0.27	<0.001
Plaque index – Site (S-PI)	0.05 ± 0.10	1.50 ± 0.38	<0.001
Gingival index – Full mouth (F-GI)	0.04 ± 0.05	1.61 ± 0.37	<0.001
Gingival index- Site (S-GI)	0	1.73 ± 0.43	<0.001
Probing depth – Full mouth (F-PD)	1.45 ± 0.19	4.50 ± 1.06	<0.001
Probing depth – Site (S-PD)	1.49 ± 0.38	6.44 ± 1.94	<0.001
CAL – Full mouth (F-CAL)	0	5.16 ± 1.09	<0.001
CAL – Site (S-CAL)	0	7.97 ± 2.65	<0.001

TABLE 2 Comparison of sulfiredoxin levels between healthy and chronic periodontitis groups using independent t-test

Group	n	Sulfiredoxin (ng/mL) Mean ± SD	t-value	*P*-value
Healthy Control	15	131.20 ± 22.88	5.44	<0.001*
Chronic Periodontitis	15	171.20 ± 16.97		

*$P < 0.05$, statistically significant.

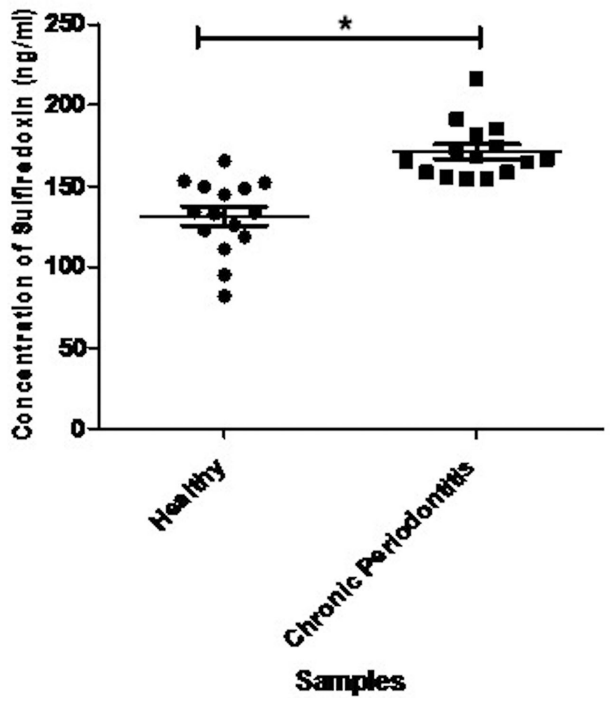

FIGURE 1 Scatter plot showing the difference in sulfiredoxin levels between the two groups with *$P < 0.001$

the test for multicollinearity was performed among the variables S-PD and age in all the subjects (n = 30) and it was found that only S-PD was emerging as a best predictor for Srx ($P = 0.001$). Also, there is no evidence of multicollinearity between age and S-PD with variance inflation factor (VIF < 5) (Table 5).

TABLE 3 Pearson correlation test between clinical parameters and sulfiredoxin levels

Variables		Group A (n = 15)	Group B (n = 15)
F-PI	r	−0.23	−0.40
	P-value	0.42	0.14
S-PI	r	0.32	−0.39
	P-value	0.25	0.15
F-GI	r	−0.34	−0.37
	P-value	0.22	0.17
S-GI	r	NA	−0.47
	P-value	NA	0.07
F-PD	r	−0.15	0.22
	P-value	0.58	0.43
S-PD	r	0.17	0.67
	P-value	0.54	0.007*
F-CAL	r	NA	0.23
	P-value	NA	0.42
S-CAL	r	NA	0.55
	P-value	NA	0.035*

**P*-value < 0.05, statistically significant; r: Pearson correlation coefficient.

4 | DISCUSSION

Sulfiredoxin has been demonstrated as an exclusive enzyme responsible for the activation of 2-Cys peroxiredoxins, which in turn are essential for scavenging the highly noxious hydrogen peroxide species. Induction of sulfiredoxin expression through the transcription factor Nrf2 (nuclear factor-erythroid2-related factor 2) was observed in various cell types.[35,36] Literature review has elucidated the role of sulfiredoxin in various oxidative stress-induced conditions and

TABLE 4 MLR analysis of site-specific clinical parameters with sulfiredoxin levels in patients with chronic periodontitis

	Regression coefficients		95% CI for B			
Predictors	**ß coefficient**	**SE**	**Lower**	**Upper**	**R^2**	***P*-value**
Constant	174.55				0.54	
S-PD	9.32	4.51	−0.73	19.37		0.06
S-CAL	−4.26	3.69	−12.49	3.97		0.27
S-GI	−12.10	11.54	−37.83	13.63		0.31
S-PI	−5.62	11.25	−30.71	19.45		0.62

MLR: multiple linear regression analysis; *P*-value < 0.05, statistically significant

TABLE 5 MLR analysis with test for multicollinearity among the variables site-specific probing depth (S-PD) and Age

	Regression coefficients		95% CI for B			Collinearity statistics
Predictors	**ß coefficient**	**SE**	**Lower**	**Upper**	***P*-value**	**VIF**
Constant	116.33					
S-PD	7.01	1.79	3.34	10.68	0.001*	2.17
Age	0.21	0.49	-0.79	1.22	0.66	2.17

VIF: variance inflation factor; **P*-value < 0.05, statistically significant

thereby ascertained the pivotal role of sulfiredoxin in the redox balance.[17]

Only a few antioxidant systems such as superoxide dismutase, catalase, and glutathione were the focus of periodontal research over the years. Sulfiredoxin was discovered in 1994, and the sulfiredoxin-peroxiredoxin antioxidant system has received a great deal of attention in systemic diseases over the recent years.[37] Various studies have demonstrated Prx's ability to not only protect against hydrogen peroxide-mediated cell death, but also their involvement in the signaling pathways that regulate cell proliferation, differentiation, and inflammation.[38,39] Therefore, it was hypothesized that the enzyme sulfiredoxin might play a role in periodontitis. The aim of the present study was to compare and estimate the levels of Srx in chronic periodontitis patients and healthy controls.

From a sample size of 35, five samples were discarded from the study due to highly diluted protein concentrations as observed using the Lowry protein estimation. In the current study, levels of the redox protein sulfiredoxin were significantly higher in diseased sites of chronic periodontitis patients than in healthy controls. Periodontitis occurs as a result of host-bacterial interaction and lipopolysaccharide (LPS) is an integral component of Gram-negative bacteria which constitute the majority of periodontopathogens. Studies showed that there is an upregulation of sulfiredoxin, both at the mRNA and protein levels, when mouse bone-marrow derived macrophages were treated with LPS.[23,40] Diet et al also showed that physiologically produced nitric oxide exerted an antioxidant effect by the induction of peroxiredoxin paralleled by an increase in the levels of sulfiredoxin, thereby playing a bridge between host defense and cell redox signaling.[41] Based on the above findings and the results of the present study, it was concluded that the elevated levels of sulfiredoxin in the tissue samples of periodontitis patients might be due to its secretion by immunostimulated macrophages as a defense mechanism against bacterial invasion.

Gingival biopsies were utilized in this study as it was suggested in earlier reports that the evaluation of antioxidant profiles in GCF and/or gingival tissues would be better to elucidate the actual role of antioxidant enzymes in the pathogenesis of periodontal diseases, instead of saliva and plasma.[42] A study conducted on gingival tissue samples of patients with type 2 diabetes with/without periodontitis and healthy controls quantified antioxidants such as Prx 1, Prx 2 using real-time polymerase chain reaction. Prx 1 gene was significantly upregulated in periodontitis patients and it was not influenced by the diabetic status whereas Prx 2 gene was slightly influenced by periodontitis.[18] The findings of the present study were concurrent with the above-mentioned findings since the authors observed an elevation in Prx levels in periodontitis patients, which in turn was negotiated, by an increase in sulfiredoxin levels. A moderate positive correlation between S-PD and S-CAL with sulfiredoxin levels were obtained using Pearson correlation test in this study. In a study by Wei et al, it was observed that there was a positive correlation between the total oxidant status and the clinical parameters such as probing depth and clinical attachment level in patients with chronic periodontitis.[43] From the above-mentioned findings, it might be speculated that there is an increased ROS activity at the diseased sites. The increased ROS levels might augment the production of antioxidants such as sulfiredoxin to neutralize the oxidative burden on the periodontal tissues. The major limitation of the present study was that the local ROS activity (H_2O_2) was not measured at the diseased sites. However, future research could be directed towards the measurement of total oxidant status, total antioxidant status along with Srx levels to determine the redox balance.

It was paradoxical to note an elevated level of antioxidants in chronic periodontitis patients, due to the fact that in an oxidative stress-mediated disease condition, it was assumed that a diminished level of antioxidants resulted in cellular injury and subsequent tissue damage. A study by Panjamurthy et al. showed that in the plasma, erythrocyte lysates and gingival tissues of periodontitis patients, there was a significant increase in enzymatic antioxidants like SOD, CAT, and Gpx when compared with the healthy controls.[21] Similar results were observed in a study by Akalin et al. where SOD levels were the highest in patients with chronic periodontitis. Patel et al. showed that Gpx was elevated in CP patients when compared with healthy controls.[44,45] So, in the present study, the increased levels of sulfiredoxin might be a defense mechanism to counteract the increased ROS activity at the local site in patients with chronic periodontitis.

Existing evidence suggested that osteoclastogenesis occurred in the milieu of reactive oxygen species, where RANK-RANKL interaction triggered a signaling cascade.[46] Further, the cascade caused an upregulation of osteoclastic genes such as tartrate-resistant acid phosphatase (TRAP) and cathepsin-K.[46] In an experimental animal study, plant-derived products such as sulforaphane and curcumin inhibited osteoclastic differentiation through activation of Nrf2 and increased the expression of their target gene, sulfiredoxin.[47] The elevated levels of sulfiredoxin in the gingival tissues of chronic periodontitis patients observed in the present study might be attributed to the development of a protective and adaptive cellular mechanism against the increased ROS production during osteoclastogenesis. The above-mentioned mechanism was the principal cause of the alveolar bone loss and eventual tooth loss in periodontal disease.[48] Nevertheless, it must be noted that there is a multitude of factors in the etiopathogenesis of periodontal disease and therefore, singling out one molecule cannot yield enough data on the same.

The present study was unique in attempting to quantify the levels of sulfiredoxin for the first time. The achieved power of the current study was 99.9% as computed by the post-hoc test.* The cases and controls in the present study were not age-matched which could be a potential limitation of the study. However, the results from the regression analysis with multicollinearity test show that age could not have been potential confounding factor on Srx levels. The peroxiredoxin-sulfiredoxin system remains as the least investigated antioxidant system in periodontal disease. Future studies aimed at providing molecular insights on the etiopathogenesis should encompass all the enzymatic antioxidants along with oxidative stress markers in both healthy and diseased states.

* G*power v3.1.9.2, Heinrich Heine University, Dusseldorf, Germany

5 | CONCLUSIONS

The present study observed that gingival tissue sulfiredoxin levels were higher in the periodontal pockets of patients with chronic periodontitis than that of healthy controls. Also, there was a positive correlation with the sulfiredoxin levels and the site-specific clinical parameters such as probing depth and clinical attachment level. The future scope of research might be directed towards validating sulfiredoxin as a biomarker and a potential target drug, which in turn could open new vistas in periodontal research.

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