Original Article

Comparison of Oxidant Stress Levels among Healthy, Chronic Periodontitis, and Ischemic Heart Disease Subjects with Presence or Absence of Chronic Periodontitis

Abstract

Objective: To investigate the total oxidant levels in healthy, chronic periodontitis (CP), and ischemic heart disease (IHD) and to check for any correlation among them. **Materials and Methods:** A sample of 80 were split into four groups of healthy subjects (Group I), CP subjects (Group II), IHD subjects (Group III), and IHD subjects having periodontitis (Group IV). The serum and saliva samples collected were analyzed for levels of hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), nitric oxide (NO), and superoxide radical (O_2^-). **Results:** There were significant ($P < 0.05$) variances in the mean serum and salivary levels of hydrogen peroxide, hydroxyl radical, NO, and superoxide within the 4 groups. Oxidant levels of both serum and saliva were lower in disease groups of Group II, III, and IV as compared to healthy controls, with different patterns. **Conclusion:** The oxidant levels (H_2O_2, OH^-, NO, and O_2^-) are significantly hampered in periodontitis and IHD subjects as compared to healthy subjects. The oxidants, whether serum or salivary, did not always show the proportional change as a result of change in oxidant stress due to disease as positive correlation was observed only in the serum H_2O_2 and salivary NO radical levels and between serum superoxide dismutase radical and salivary H_2O_2 in Group I. In Group III, there was a positive correlation between serum NO radical and salivary H_2O_2.

Keywords: *Oxidant stress, periosystemic interlink, reactive oxygen species*

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Introduction

Periodontium is an investing and supporting tissue of human dentition. Periodontal diseases are some of the oldest and most common diseases which cause soft and hard tissue destruction ultimately leading to tooth loss.[1]

Chronic infection and inflammation are an underlying feature of periodontal disease and the same are presently being thought of as new risk factors for atherosclerotic cardiovascular disease (CVD).[1-3] Studies revealed that periodontal disease has the capacity to increase the inflammatory burden which can amplify and kick-start the process of atherosclerosis.[3] Increased levels of oxidants/reactive oxygen species (ROS) leads to a state of oxidative stress and has been found to be connected with the unfolding mechanism of a large number of chronic diseases, such as cardiovascular and periodontal diseases.[4,5]

Oxygen is crucial for function because it is indispensable for many metabolic functions, which include respiration at cellular level, but is deleterious for the cells when present in reactive form.[6,7] Oxidants/ROS are one such reactive and unstable species which in excess can create havoc in exacerbating periodontal disease by creating a state of oxidant stress when in excess. These results could put forward an idea that periodontal therapy may affect local ROS production, which would, in turn, alter the oxidative state at the systemic level.[8] In turn, this host discomposure could represent one of the connecting links and the association between periodontal therapy and vascular function changes.[9] Oxidants/ROS are highly reactive and have been identified as important signaling molecules in various cellular processes.[6] Molecular oxygen is the source for ROS and are capable to damage the proteins, lipids, and deoxyribonucleic acid if not neutralized by antioxidant substances.[6] Oxidants can exist in radical forms such as superoxide (O2•–),

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hydroxyl (OH•), alkoxyl (LO•, R–O•), peroxyl (LOO•, ROO•), and nitric oxide (NO•) or in nonradical forms such as peroxynitrite (ONOO–), hypochlorite ($HOCl^-$), hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^1O_2$), and hydro peroxide (ROOH).[7] Toxic compounds, tobacco smoke, chemical/enzymatic reactions, ultraviolet, and ionizing radiation can also produce ROS in the cells.[10,11]

Only handful have studied the relationship between oxidant stress and periodontitis.[12-14] In many pathologic conditions (including heart and vascular diseases), total oxidant capacity or any corresponding change in antioxidants (AOs) could be a reliable biomarker of diagnostics and prognostics.[15,16]

Hence, the aim of this study was to estimate salivary and serum levels of total oxidants, superoxide radical, hydroxyl radical, NO, and hydrogen peroxide in periodontal health, periodontal disease and to correlate the levels with ischemic heart disease (IHD) subjects.

Materials and Methods

The research protocol was approved by the Ethical Committee of A.B. Shetty Memorial Institute of Dental Sciences, NITTE deemed to be University and was conducted in accordance with the ethical regulations of the Declaration of Helsinki. Subjects were included from the outpatient department of Periodontics, A.B. Shetty Memorial Institute of Dental Sciences, Deralakatte, Mangalore, Karnataka, and department of Cardiology K.S. Hegde Medical Academy, Deralakatte, Mangalore, Karnataka, India. Final sample of 80 subjects were included based on the selection criteria. Later, subjects were categorized into the following four groups of 20 participants each based on screening examination. Group I included subjects who were systemically and periodontally healthy, Group II comprised subjects with chronic periodontitis (CP), Group III comprised subjects with IHD without periodontitis (IHD-CP), and group IV comprised subjects with IHD and periodontitis (IHD + CP). Written informed consent was obtained from all subjects at the beginning of the study. Complete case history was carried out for all subjects.

As per the inclusion criteria, subjects between the age group of 25 and 65 years, having at least 20 teeth, in good systemic and periodontal health for Group I and Group III, were included. Subjects having signs and symptoms of ischemia, electrocardiogram changes which may include pathologic Q waves, ST elevation, or depression, and change in biochemical markers such as cardiac troponins and creatinine phosphokinase-MB were diagnosed as a case of IHD[17,18] (Group III and Group IV). Subjects with either moderate or severe CP (Group II and IV) were included, based on the detection of clinical attachment level (CAL) ≥3 mm, interdental CAL ≥2 mm, and probing depth ≥4 mm in more than 30% of sites measured or pocket probing depth (PPD) ≥3 mm in ≥2 teeth of nonadjacent teeth with a William's periodontal probe and interdental CAL of ≥2 nonadjacent teeth with pocketing >3 mm is detectable at ≥2 teeth. The CAL should not be attributed to nonperiodontal causes of trauma-induced gingival recession, cervical dental caries, etc.[19,20] The Michigan O probe with William's markings (William's periodontal probe) was used as the missing marking of 4 and 6 mm makes visualization of the pocket depth easy for the examiner.[21]

Subjects with a history of smoking, tobacco consumption, and any anti-inflammatory/antibiotic therapy for 6 months erstwhile to study and those on minerals/vitamin or AOs supplement intake during the past 3 months were not included in the study. In addition, lactating/pregnant women and subjects diagnosed with systemic disease or conditions aside from IHD (for Group III and Group IV) were also excluded. Next, the subjects were screened which included recording of gingival index,[22] PPD, and CAL.

Sample collection

All measurements of recording were done before collection of sample. The subjects were instructed not to consume any fluids or food or brush teeth for at least 30 min before sampling. Unstimulated whole saliva and venous blood of 5 mL were collected. Blood was drawn from the median cubital vein, which is superficial and easily accessible by venepuncture and was transferred in blood collection plastic tubes which were plain and ethylenediaminetetraacetic acid coated. The samples were sent for biochemical analysis for the estimation of total oxidant levels, hydroxyl radical, superoxide radical, hydrogen peroxide, and NO. All the samples were analyzed on the same day of collection.

Biochemical analysis

The levels of hydrogen peroxide, hydroxyl radical, NO, and superoxide were estimated using the methods by Ruch *et al*., Elizabeth and Rao, Green *et al*., and Winterbourn *et al*. using a spectrophotometer (Genesys 10-S, USA) at wavelength of 230 nm, 532 nm, 546 nm, and 560 nm. The detailed description regarding the reagents used and procedure of each method for the estimation of each radical is provided separately [Attached as Supplementary Data].

Statistical analysis

Descriptive statistics of the parameters studied were calculated using IBM SPSS statistical software version 22 (Armonk, NY, USA: IBM Corp) software and presented with suitable diagrams and graphs. For comparing oxidant levels between four groups of healthy (Group I), subjects with CP (Group II), IHD subjects without periodontitis (Group III), and IHD subjects with periodontitis (Group IV), one-way analysis of variance (ANOVA) was used. Pairwise comparison was

done using Tukey's *post hoc* test. Pearson's correlation coefficient was applied to find any correlation between the oxidant levels in serum and saliva. The data were expressed as mean ± standard deviation. Level of significance for this study was 5% with probability value of <0.05 being considered as statistically significant.

Results

Among the 80 subjects included in the study, 31.25% were female, whereas 68.75% were male. The mean age of the subjects included in the study was 47.3 years. The descriptive parameters of mean periodontal pocket depth and clinical attachment loss of the subjects in each group are described in Table 1. The mean oxidant levels in serum and saliva of subjects in Group I to IV are represented in Figures 1 and 2 and Tables 2 and 3. ANOVA test was used for comparison of serum levels of ROS such as hydroxyl radical, NO, hydrogen peroxide, and superoxide radical. The results revealed that there were statistically significant differences among the groups with $P < 0.001$ in both the serum and salivary oxidants/ROS. The oxidant levels showed the highest values in Group IV, followed by Group II, Group III, and Group I in decreasing order. Pairwise comparison was done using Tukey's *post hoc* test of serum and salivary values which again showed statistically significant differences with P value <0.001*, confirming that the difference is significant and not by chance. The results of this analysis are included in a supplementary file mentioned as Supplementary Table 1 for pairwise comparison of serum oxidants and Supplementary Table 2 for pairwise comparison of salivary oxidants.

Table 1: Comparison of clinical attachment loss and periodontal pocket depth between the groups

Parameters	Group I	Group II	Group III	Group IV
Clinical attachment loss (mm)	2.24±0.1	4.70±0.14	2.24±0.1	5.40±0.24
Periodontal pocket depth (mm)	1.40±0.11	4.27±0.11	1.40±0.11	4.76±0.55

Table 2: Comparison of serum levels of reactive oxygen species

ROS	Groups	Mean±SD	*P*
Hydroxyl radical	Group I	20.15±2.82	<0.001*
	Group II	53.66±7.05	
	Group III	31.18±2.30	
	Group IV	63.68±3.91	
Superoxide radical	Group I	25.15±3.59	<0.001*
	Group II	50.26±6.06	
	Group III	34.23±1.87	
	Group IV	64.42±4.24	
NO radical	Group I	34.56±3.19	<0.001*
	Group II	66.84±12.29	
	Group III	38.81±2.57	
	Group IV	78.76±3.42	
H_2O_2 radical	Group I	24.58±2.62	<0.001*
	Group II	53.85±5.48	
	Group III	31.78±2.78	
	Group IV	63.70±6.26	

*P<0.001 statistically significant, P>0.05, NS. ROS: Reactive oxygen species, SD: Standard deviation; NS: Nonsignificant; NO: Nitric oxide; H_2O_2: Hydrogen peroxide

In order to correlate oxidant levels in saliva and serum, Pearson's correlation coefficient was used. The results [Table 4] put forward that there was a positive correlation between the serum H_2O_2 and salivary NO radical levels and between serum O_2^- radical and salivary H_2O_2 in Group I. In Group III, there was a positive correlation between serum NO radical and salivary H_2O_2. In Group IV, there was a negative correlation between serum O_2^- radical and salivary NO. On the other hand, there was no correlation observed in Group II.

Discussion

The periosystemic interlink in relation to the CVDs is based on numerous mechanisms: first is the focal spread of infection from periodontal pocket wound to systemic circulation via bacteremia reaching the endothelium; second is the spread of inflammation from periodontal pocket to the systemic circulation via activation of inflammatory mediators; the third mechanism involves an autoimmune response of the body to the endothelium due to the homology in the heat shock protein of endothelium and periodontal pathogens; and the final mechanism is the role of increased oxidant stress [Figure 3] in initiating the endothelial damage by decreasing NO availability, which results in endothelial dysfunction contributing toward atherosclerotic disease process and subsequent ischemic changes.[3,23,24]

The present study was directed to understand whether the level of oxidants could be used to further strengthen the evidence of periosystemic interlink between CP and IHD. In this study, we compared serum and salivary levels of oxidants, such as hydroxyl radical, NO, hydrogen peroxide, and superoxide radical which showed significant ($P < 0.05$) difference in their mean values in the four study groups. These results are comparable with the reports published by Chapple and Matthews, 2007, on saliva and serum levels of ROS and antioxidant molecules in subjects diagnosed with CP.[8,14]

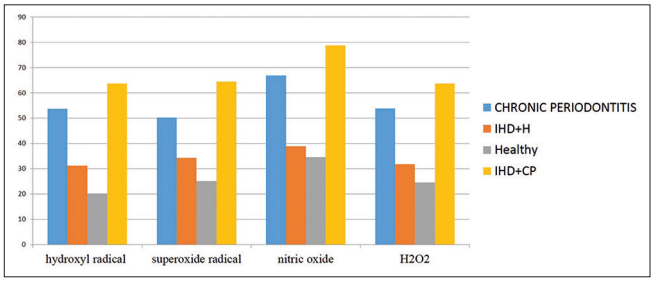

Figure 1: Bar graph showing the serum oxidant levels among study groups

Table 3: Comparison of salivary levels of reactive oxygen species

ROS	Groups	Mean±SD	*P*
Hydroxyl radical (U/mg Hb)	Group I	18.45±5.37	<0.001*
	Group II	35.65±3.27	
	Group III	30.35±2.18	
	Group IV	44.33±4.61	
Superoxide radical (U/mg Hb)	Group I	18.45±5.37	<0.001*
	Group II	34.88±3.26	
	Group III	24.57±2.25	
	Group IV	43.25±4.90	
NO radical (U/mg Hb)	Group I	25.14±2.26	<0.001*
	Group II	49.36±5.68	
	Group III	33.74±2.22	
	Group IV	60.39±5.42	
H_2O_2 radical (U/mg Hb)	Group I	23.21±1.97	<0.001*
	Group II	37.32±6.20	
	Group III	31.43±3.58	
	Group IV	44.76±4.93	

**P*<0.001 statistically significant, *P*>0.05, NS. NO: Nitric oxide; H_2O_2: Hydrogen peroxide; NS: Nonsignificant; ROS: Reactive oxygen species; SD: Standard deviation

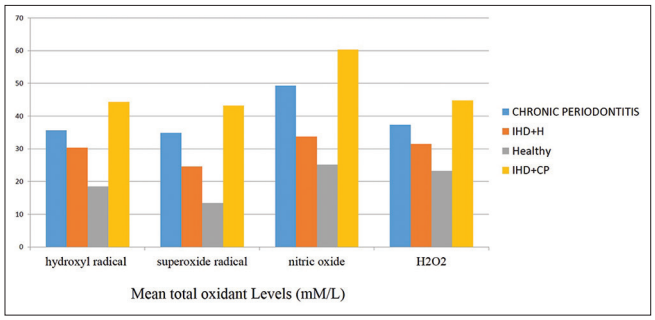

Figure 2: Bar graph showing the salivary oxidant levels among study groups

Our study results indicate that periodontitis acts as a common focal source of inflammation, which, in turn, results in a state of systemic oxidative stress.[12,15] The notion that oxidants are linked with pathogenesis of inflammatory diseases and could directly or indirectly contribute to tissue damage has become a chief area of research over the last few years. However, the proof available to substantiate the role of oxidants in tissue damage is often indirect or incidental.[25] There are few reports in literature which fulfill any, or all, of Halliwell's postulates. These postulates are similar to those given by Koch in 1884 and serve as criteria for ROS to be concluded as a key mediator of tissue injury in a given disease. According to the postulates, oxidants should be present before or at the same time at the site of injury and direct application to tissues at concentrations found *in vivo* should result in reproducible damage as seen in diseased tissue. Finally, eliminating oxidants should diminute tissue injury to the magnitude of their antioxidant action *in vivo*.[25-27]

Our results suggested that ROS increased in site of injury like periodontitis as compared to healthy group, which is in agreement with the criteria proposed by Halliwell. Papaharalambus, 2007,[28] proposed that ROS production is tightly controlled by AOs keeping the concentration of ROS in the pico molar range. This low concentration of ROS is needed for adequate cell bodily processes. When ROS is excessively produced or AOs are depleted, there is a high intracellular ROS, leading to oxidative stress and resulting in cellular damage.[28,29] Individuals suffering from periodontitis might be at higher risk of aggravating other chronic systemic inflammatory diseases, such as CVDs and diabetes,[28,29] and this consideration would help in understanding the potential role of ROS as one of the mechanisms of these associations.

The clinical parameters (CAL and PPD) indicate that the more severe the inflammation of the periodontal tissue, the higher is the level of oxidative stress in the tissues. For instance, highest CAL and PPD values were seen with Group IV compared with other groups [Table 1] Comparison of oxidant levels showed the highest values in Group IV when compared to other groups. These results reinforce the clinical and epidemiological studies which have shown that periodontal disease, a local infection of the oral cavity caused majorly by Gram-negative bacteria, is a risk factor for CVDs. ROS generation is considered to ensue during ischemia with low oxygen tension. The mechanisms which link periodontal disease to CVD are imprecise, but it is thought that periodontitis induces inflammation in the systemic compartment which contributes toward atherosclerosis by activation of biochemical reaction cascade resulting in initiation and development of plaque formation and injury of the endothelium.[29,30] This may help in explaining as to why the ROS levels were comparatively lower in Group III as compared to Group II. Tables 1 and 2 have shown that the ROS levels are maximum in IHD and periodontitis together, suggesting the increased burden of oxidant stress due to periodontal disease as compared to ROS in case of IHD alone. In addition, the ROS levels are greater in Group II than Group III, suggesting that periodontal disease may be responsible for a profound increase in ROS resulting in oxidant stress. Cellular function is modulated by ROS in CVDs. An extracellular stimuli can generate ROS enzymatically and activate resident vascular cells, leading to altered cellular function and impairment of endothelium dependent vasodilation in the coronary circulation of subjects, resulting in endothelial dysfunction and heart failure in due course.[31] In this study, we demonstrated that superoxide radical and hydroxyl radical showed statistically significant difference with *P* value (< 0.001) among the groups. Oxidants such as O_2^- and H_2O_2 may have protective actions via signaling for preconditioning or oxidant stress-induced gene products that activate multiple groups of protective proteins.[31,32] However, with ischemia and reperfusion, the normal balance is lost and OH can be produced via the Fenton reaction.[32] Key *et al*., 1990,[33] and Steinbeck *et al*., 1994,[34] suggested that ROS are produced by osteoclasts at the ruffle border and may play a role in

Table 4: Correlation between serum and salivary levels in each group of reactive oxygen species

Condition	Serum levels	Salivary levels			
		Hydroxyl radical	SOD radical	NO radical	H_2O_2 radical
Group II	Hydroxyl radical (U/mg Hb)				
	Pearson correlation	−0.032	−0.201	−0.190	0.041
	P	0.894 (NS)	0.396 (NS)	0.423 (NS)	0.863 (NS)
	Superoxide radical (U/mg Hb)				
	Pearson correlation	0.199	0.359	−0.106	−0.010
	P	0.401 (NS)	0.120 (NS)	0.656 (NS)	0.966 (NS)
	NO radical (U/mg Hb)				
	Pearson correlation	−0.153	0.100	−0.097	−0.377
	P	0.519 (NS)	0.674 (NS)	0.684 (NS)	0.102 (NS)
	H_2O_2 radical (U/mg Hb)				
	Pearson correlation	0.237	0.083	−0.246	0.053
	P	0.315 (NS)	0.727 (NS)	0.297 (NS)	0.825 (NS)
Group III	Hydroxyl radical (U/mg Hb)				
	Pearson correlation	0.265	0.246	0.007	−0.173
	P	0.258 (NS)	0.296 (NS)	0.977 (NS)	0.467 (NS)
	Superoxide radical (U/mg Hb)				
	Pearson correlation	0.379	−0.105	−0.163	0.280
	P	0.099 (NS)	0.658 (NS)	0.492 (NS)	0.232 (NS)
	NO radical (U/mg Hb)				
	Pearson correlation	0.054	0.360	−0.094	0.526
	P	0.822 (NS)	0.119 (NS)	0.692 (NS)	0.017*
	H_2O_2 radical (U/mg Hb)				
	Pearson correlation	−0.204	−0.167	−0.281	−0.408
	P	0.388 (NS)	0.481 (NS)	0.231 (NS)	0.074 (NS)
Group I	Hydroxyl radical (U/mg Hb)				
	Pearson correlation	−0.111	0.429	−0.170	0.321
	P	0.640 (NS)	0.059 (NS)	0.473 (NS)	0.168 (NS)
	Superoxide radical (U/mg Hb)				
	Pearson correlation	−0.153	0.061	0.142	0.449
	P	0.520 (NS)	0.798 (NS)	0.551 (NS)	0.047*
	NO radical (U/mg Hb)				
	Pearson correlation	0.209	0.412	0.354	0.376
	P	0.377 (NS)	0.071 (NS)	0.126 (NS)	0.102 (NS)
	H_2O_2 radical (U/mg Hb)				
	Pearson correlation	0.000	−0.100	0.488	−0.134
	P	1.000 (NS)	0.676 (NS)	0.029*	0.573 (NS)
Group IV	Hydroxyl radical (U/mg Hb)				
	Pearson correlation	−0.380	−0.175	0.022	0.256
	P	0.098 (NS)	0.460 (NS)	0.928 (NS)	0.276 (NS)
	Superoxide radical (U/mg Hb)				
	Pearson correlation	0.117	−0.173	−0.523	−0.084
	P	0.624 (NS)	0.465 (NS)	0.018*	0.724 (NS)
	NO radical (U/mg Hb)				
	Pearson correlation	0.077	0.347	−0.023	0.384
	P	0.746 (NS)	0.134 (NS)	0.924 (NS)	0.095 (NS)
	H_2O_2 radical (U/mg Hb)				
	Pearson correlation	−0.061	0.354	0.123	−0.299
	P	0.799 (NS)	0.125 (NS)	0.604 (NS)	0.200 (NS)

*$P<0.05$ statistically significant, $P>0.05$, NS Correlation of levels in saliva and serum did not show statistical significant result with $P>0.05$. NS: Nonsignificant; NO: Nitric oxide; H_2O_2: Hydrogen peroxide; SOD: Superoxide dismutase

resorption and certain ROS, such as O_2^- and H_2O_2, play a role in the activation of osteoclasts, rather than in the direct degradation of the bone matrix.[5,33,35,36]

The statistically significant positive correlation values observed [Table 4] in Group I and III and negative correlation in Group IV could suggest that the H_2O_2 and

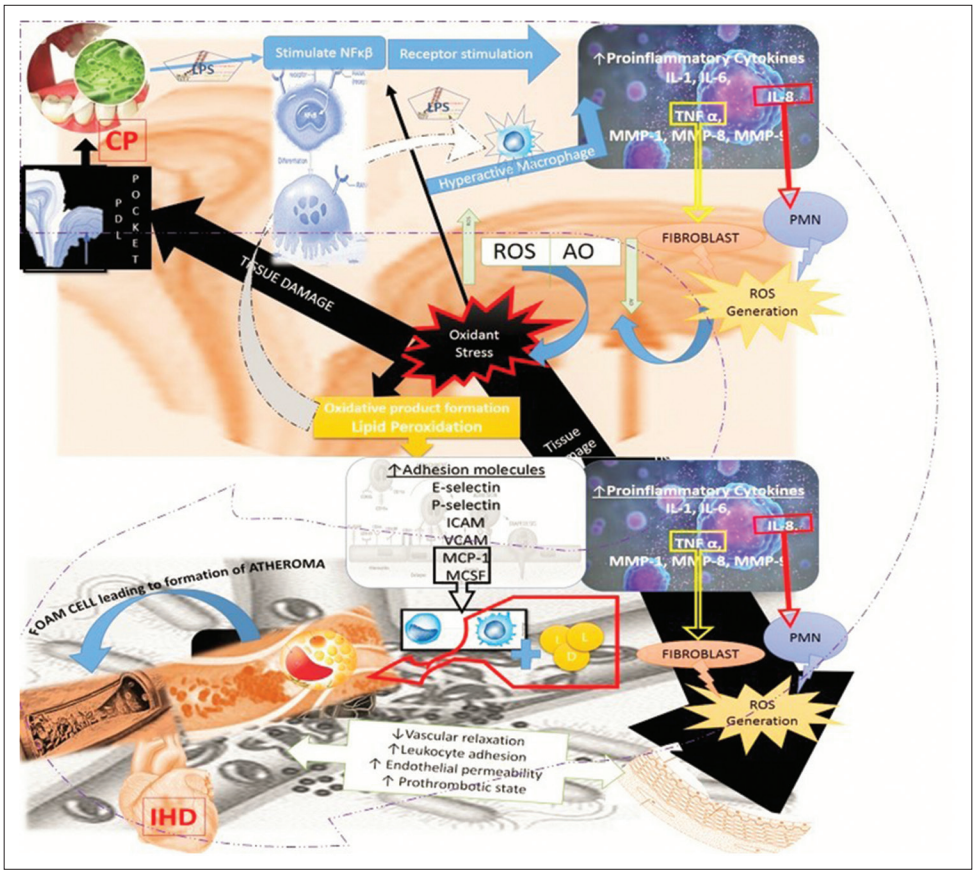

Figure 3: Bacteria and lipopolysaccharide in periodontal pocket spread directly via blood stream and indirectly via interleukins and reactive oxygen species which results in lipid peroxidation ↑ adhesion molecule intercellular adhesion molecule, vascular cell adhesion molecule. Monocyte chemoattractant protein-1 and macrophage colony stimulating factor attracting monocytes and convert to macrophages. Reactive oxygen species ↓ nitric oxide in endothethelium -> ↓vascular relaxation, ↑PMN adhesion and prothrombotic state. Macrophages + low density lipoprotein -> foam cells and initiate ischemic heart disease

NO radicals produced locally in oral cavity might affect the NO and H_2O_2 radicals produced in serum and vice versa in health as well as in systemic disease such as IHD ± CP. Although the exact mechanism of how these interactions occur and their clinical implication is not well understood or established, the results pose a ground for future research into the interactions between various radicals.

Conclusion

Oxidative stress depicted by increased serum and salivary oxidants in CP subjects and in subjects having both CP and IHD as compared to healthy controls was observed in the present study. However, the correlation between the serum and salivary oxidants did not follow the same course and requires further research to understand the further correlation of vascular measures with oxidative stress after periodontal therapy to confirm the same.

Limitations

The main limitations of the study include a small sample size and the inherent limitations of the study design. Further longitudinal studies of larger stratified populations will be required to validate the bearing of periodontal disease and treatment on systemic oxidative stress. As the study was carried out before 2017, the case definition by Armitage was used for inclusion, but on retrospective examination of the data of subjects, the case definition provided by 2017 classification is applicable to the cases which are included in the present study.

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Conflicts of interest

There are no conflicts of interest.

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Supplementary Table 1: Pairwise comparison of serum oxidants using Tukey's *post hoc* test

Dependent variable	Condition (I)	Condition (J)	Mean difference (I-J)	SE	95% CI		*P*
					Lower	Upper	
Hydroxyl radical	Group II	Group III	22.47	1.39	18.80	26.15	<0.001*
		Group I	33.51	1.39	29.83	37.18	<0.001*
		Group IV	−10.02	1.39	−13.69	−6.34	<0.001*
	Group III	Group I	11.03	1.39	7.35	14.70	<0.001*
		Group IV	−32.49	1.39	−36.17	−28.82	<0.001*
	Group I	Group IV	−43.53	1.39	−47.20	−39.85	<0.001*
Superoxide radical	Group II	Group III	16.03	1.33	12.52	19.53	<0.001*
		Group I	25.11	1.33	21.60	28.61	<0.001*
		Group IV	−14.15	1.33	−17.65	−10.64	<0.001*
	Group III	Group I	9.07	1.33	5.57	12.58	<0.001*
		Group IV	−30.18	1.33	−33.69	−26.67	<0.001*
	Group I	Group IV	−39.26	1.33	−42.77	−35.75	<0.001*
NO radical	Group II	Group III	28.02	2.11	22.45	33.59	<0.001*
		Group I	32.28	2.11	26.71	37.84	<0.001*
		Group IV	−11.91	2.11	−17.48	−6.35	<0.001*
	Group III	Group I	4.25	2.11	−1.31	9.82	0.194 (NS)
		Group IV	−39.94	2.11	−45.51	−34.37	<0.001*
	Group I	Group IV	−44.19	2.11	−49.76	−38.63	<0.001*
H_2O_2 radical	Group II	Group III	22.064	1.44	18.25	25.87	<0.001*
		Group I	29.26	1.44	25.46	33.07	<0.001*
		Group IV	−9.85	1.44	−13.66	−6.04	<0.001*
	Group III	Group I	7.20	1.44	3.39	11.01	<0.001*
		Group IV	−31.91	1.44	−35.72	−28.11	<0.001*
	Group I	Group IV	−39.12	1.44	−42.92	−35.31	<0.001*

**P*<0.001 statistically significant *P*>0.05, NS. CI: Confidence interval; NO: Nitric oxide; H_2O_2: Hydrogen peroxide; NS: Nonsignificant; SE: Standard error

Supplementary Table 2: Pairwise comparison of salivary oxidants using Tukey's *post hoc* test

Dependent variable	Condition (I)	Condition (J)	Mean Difference (I-J)	SE	95% CI		*P*
					Lower	Upper	
Hydroxyl radical	Group II	Group III	5.29	1.28	1.92	8.65	0.001*
		Group I	17.19	1.28	13.82	20.56	<0.001*
		Group IV	−8.68	1.28	−12.05	−5.31	<0.001*
	Group III	Group I	11.90	1.28	8.53	15.26	<0.001*
		Group IV	−13.97	1.28	−17.34	−10.61	<0.001*
	Group I	Group IV	−25.87	1.28	−29.24	−22.51	<0.001*
Superoxide radical	Group II	Group III	10.30	1.04	7.55	13.05	<0.001*
		Group I	21.49	1.04	18.74	24.24	<0.001*
		Group IV	−8.36	1.04	−11.11	−5.61	<0.001*
	Group III	Group I	11.19	1.04	8.44	13.94	<0.001*
		Group IV	−18.67	1.04	−21.42	−15.92	<0.001*
	Group I	Group IV	−29.86	1.04	−32.61	−27.11	<0.001*
NO radical	Group II	Group III	15.62	1.34	12.10	19.14	<0.001*
		Group I	24.22	1.34	20.70	27.74	<0.001*
		Group IV	−11.03	1.34	−14.55	−7.51	<0.001*
	Group III	Group I	8.59	1.34	5.07	12.11	<0.001*
		Group IV	−26.65	1.34	−30.17	−23.13	<0.001*
	Group I	Group IV	−35.25	1.34	−38.77	−31.73	<0.001*
H_2O_2 radical	Group II	Group III	5.88	1.41	2.17	9.59	<0.001*
		Group I	14.10	1.41	10.39	17.81	<0.001*
		Group IV	−7.44	1.41	−11.14	−3.73	<0.001*
	Group III	Group I	8.22	1.41	4.51	11.92	<0.001*
		Group IV	−13.32	1.41	−17.03	−9.62	<0.001*
	Group I	Group IV	−21.54	1.41	−25.25	−17.84	<0.001*

**P*<0.001 statistically significant, *P*>0.05, NS. CI: Confidence interval; NO: Nitric oxide; H_2O_2: Hydrogen peroxide; NS: Nonsignificant; SE: Standard error