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Research Article

COMPARATIVE ESTIMATION OF SALIVARY PROTEIN CARBONYL LEVELS AMONG SMOKERS AND SMOKELESS TOBACCO CHEWERS WITH CHRONIC PERIODONTITIS. A CROSS SECTIONAL CLINICO-BIOCHEMICAL STUDY

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ABSTRACT

AIM; To determine salivary protein carbonyl (PC)levels among smokers and smokeless tobacco (ST) chewers with chronic periodontitis(CP). **MATERIAL AND METHODS;** A total number of 120 subjects were selected based on their clinical parameters into FOUR GROUPS: Group 1 (30 healthy subjects), Group 2 (30 CP subjects), and Group 3 (30 CP SMOKERS) Goup 4 (30 CP Smokeless Tobacco chewers). Saliva samples were collected. PC levels were estimated according to method described by Levine et al. **RESULTS :** The PC concentration in saliva was highest in subjects with CPS as compared to other three groups, where as the PC concentartion was also found to be higher in saliva of CPST as compared with healthy and CP groups but marginally lesser than CPS, and a positive association was observed between saliva PC levels and all periodontal parameters**. CONCLUSIONS ;** There was an increase in PC levels in saliva as the disease process progressed from healthy,and CP, modified by risk factors like smoking and smokeless tobacco chewing suggesting a role for increased oxidative stress in CP along with the deleterious effects of smoking and smokeless tobacco chewing on periodontium.

1. INTRODUCTION

Translational application of experimental knowledge to the clinical setting is the ultimate goal of biomedical research. Periodontitis is a polymicrobial infection induce inflammatory process that gradually, if undiagnosed and untreated, leads to irreversible periodontal soft and hard-tissue destruction and finally to tooth loss[2, 18]. Periodontitis being multifactorial disease entity has also been implicated with increased oxidative stress in the pathogenesis of periodontitis [27, 10].

Oxidative stress is a state of altered physiological equilibrium within a cell or tissue/organ, defined as "a condition arising when there is a serious imbalance between the levels of free radicals in a cell and its antioxidant defences in favour of the former[13]. It is estimated that 1-3 billion reactive oxygen species (ROS) are generated/cell/day, and given this, the importance of the body's antioxidant defense systems to the maintenance of health becomes clear[8].

ROS is a term that has become more popular because it encompasses other reactive species

which are not true radicals but are nevertheless capable of radical formation in the intra- and extracellular environments[19].

Oxidative modifications of enzymes and structural proteins play a significant role in the aetiology and/or progression of several human diseases[26]. Carbonylation is an irreversible, non-enzymatic modification of proteins.ROS can react directly with protein or they can react with molecules such as sugars and lipids, generating products that then react with protein and lead to the formation of protein carbonyl(PC) derivatives. ROS induced protein modifications can result in unfolding or alteration in protein structure[26, 31, 32].

Cigarette smoke (CS) is rich in free radicals , and it can also accelerate the production of ROS by recruiting and activating phagocytes in the lung. Thus it is widely believed that at least some of the deleterious effects of CS involve oxidative damage[13].

The biological plausibility of the increased periodontal disease severity and rate of progression associated with smoking has been hypothesized to be due to interactions among smoking, bacterial periodontal pathogens, and the host[30]. Incontrast, the potential periodontal health effects of smokeless tobacco (ST), which is also available in various forms, such as loose leaf, pouch, or snuff, have received much less attention[30]

According to Global Adult Tobacco survey conducted by Ministry of Health and Family Welfare Government of India 2009-10, the prevalence of current tobacco use in any form in Indian population was found to be 34.6% of adults, out of which current tobacco smokers were 14% of adults and current smokeless tobacco users were 25.9%[39]. In light of these findings the effects of ST chewing on periodontal health can not be neglected.

Smoking and ST chewing being the most important risk factors associated with periodontitis[41] . The use of ST has also shown its contribution in loss of clinical attachment level (CAL) along with local gingival recession at the site of placement[30, 10].

Saliva, the first biological fluid to encounter inhaled cigarette smoke or chewed ST is known for its highly protective functions against deleterious agents such as microorganisms, toxins, and various oxidants[22, 4].

Indeed measurement of PCs has been used as a sensitive assay for oxidative damage to proteins, partly because it measures several different consequences of oxidative damage induced by multiple forms of ROS [33, 42].

Therefore, this cross sectional clinico biochemical investigation was done to compare the PC levels as an important marker of protein oxidation in saliva in clinically healthy periodontium , chronic periodontitis, smokers and smokeless tobacco chewers with chronic periodontitis.

MATERIALS AND METHODS

1. Study design participants and groupings

One hundred and twenty patients attending for routine dental examination at Outpatient Department of Periodontics, P.M.N.M dental college and Hospital Bagalkot, Karnataka (INDIA) were recruited to the study. The age range of the subjects was 17-65. Those taking nutritional supplements and antibiotics were excluded from the study. Also excluded were those having systemic diseases like diabetes mellitus, hepatitis, cancer and HIV infection.

All participants gave their written informed consent for inclusion in the study.

Recruitment took place between September 2013 and February 2014 .Ethical approval was obtained from institutional own Ethical Committee.

According to the criteria approved by the 1999 International Workshop for the classification of periodontal diseases and conditions[18],the participants were catogorised under four groups..group A 30 subjects with healthy periodontium(H), group B 30 wth chronic periodontitis(CP), group C 30 { CP smoker, who had smoked 100 or more cigarettes over their life time.(CPS)[37]}., group D 30 {CP smokeless tobacco chewer, who had been chewing at least one sachet/pouch of tobacco daily for at least 12months (CPST) [24]}.

Clinical examination done to assess the periodontal condition included gingival index[3], CAL and pocket probing depth (PPD). Clinical assessments using the above mentioned parameters were performed by a single examiner, using a graduated William's periodontal probe.

2. Sample collection and analysis

Whole unstimulated saliva was collected in this study as it represents the major intra-oral condition regarding saliva state and composition. It also contains some elements of gingival crevicular fluid and tissue metabolites which can be useful in the determination of tissue degradation [22,14]. In addition, stimulating saliva flow has been demonstrated to increase saliva volume and disrupt the antioxidant concentration[15]. Saliva samples were collected after patients had received their routine check-up. With the patients seated, the saliva was collected over a 5-min period with instructions to allow saliva to pool in the bottom of the

mouth and drain to a collection tube when necessary.

Subjects were asked not to swallow any saliva for the duration of the collection to allow the calculation of salivary flow rates. At the end of the collection period, saliva volume was measured, the tube sealed and then frozen in dry ice until taken back to the laboratory for processing. Prior to analysis, the saliva was placed into eppendorf tubes and centrifuged at 4000 ***g*** for 10 min at 4 ◦C. The supernatant fraction was then aliquotted into storage vials and kept at −80 ◦C until required for analysis.

3. Determination of oxidative injury

PCs were estimated according to method described by ***Levine* et al**[9]. Which is highly sensitive assay contains 2,4-dinitrophenylhydrazine (DNPH), which reacts with PCs forming a Schiff base to produce the 2,4-

DNPH product measure spectrophotometrically at 370nm. The concentration of PCs was expressed as nmol/mg of total protein/ml of saliva determined by the mathematical formula;

PC(nmolcarbonyl/mg total protein/ml)= {(CA)/(*.011µM^{-1})}(500µl/200µl)

CA- average Corrected Absorbance, *the actual extinction coefficient for dinitrophenylhydrazine at 370 nm is 22000 M^{-1} ($0.22\mu M^{-1}cm^{-1}$). This value has been adjusted for the pathlength of the solution in the well.

4. Statistical analysis

The data were analyzed using statistical software SPSS (version 15).Analysis of variance (ANOVA) and Tukeys multiple post hoc comparison test were carried out for comparison of salivary levels of PCs between the groups. To evaluate the baseline mean GI scores, Kruskal Wallis ANOVA was used, followed by pairwise comparison among the groups with Mann Whitney U test. Also for PPD and CAL scores among all the four groups, ANOVA followed by tukeys multiple post hoc procedure was done. $P < 0.05$ were considered statistically significant. Using Pearson's correlation coefficient, the relationship between salivary PC levels and the clinical parameters were analyzed.

$P < 0.05$ were considered statistically significant.

RESULTS

The descriptive statistics along with the mean ± SD of age, sex, GI, PPD, and CAL (of all groups) are tabulated in Table 1.

All the samples in each group tested positive for PC. Table 2 , shows the mean PC levels in saliva as highest for Group III (CS) **173.18 _+29.68**, followed by Group IV (CPST) **156.62 +31.99** and Group II (CP) **102.78+18.17,** and least values were found in Group I (H) **32.03+7.14**. When intergroup comparison was done among the four groups , the differences in the mean salivary PC levels were found to be stasticaly significant. Pearson correlation coefficients between the clinical parameters and PC levels are tabulated in Table 3. The positive correlation was found between PC levels and PPD in all the groups except group 4. However the correlation between PC Levels and CAL was found to be positive in all the groups but values were not statistically significant. GI was found to be significant only in Group 4 and weak positive (not statistically significant at $P > 0.05$) correlation in Group 3.

Table 1: Descriptive statistics of study population (mean±SD)

Table 2: Results of analysis of variance and pair-wise comparison using Tukeys multiple post hoc test of the mean salivary protein carbonyl levels between the four groups

Table 3: Relationship of salivary protein carbonyl levels to clinical parameters

DISCUSSION

Tobacco consumption is one such habit that is ironically referred to as an "aromatic means of lining the pocket of the manufacturer, the lungs of the consumer and the boxes of the undertaker".IIn the present study we report for the first time, levels of PCs in saliva of smokers and ST consumers with CP.

The demonstration that oxidatively modified forms of proteins accumulate during oxidative stress and in some pathological conditions has focused attention on physiological and non-physiological mechanisms for the generation of ROS [1], and on the modification of biological molecules by various kinds of ROS. Basic principles that govern the oxidation of proteins by ROS were established in the pioneering studies [1,2,6,7] that characterized reaction products formed when proteins were exposed to ionizing radiation under conditions where only zOH, O2 ., or a mixture of both was made available. Collectively ROS can lead to oxidation of amino acid residue side chains, formation of protein-protein cross-linkages, and oxidation of the protein backbone resulting in protein fragmentation.[2,7]

The PC content resulted by oxidation makes the protein resistant to hydrolysis and functional inactivation of proteins in serum or plasma, cellular components, membrane proteins etc.

since, protein is major constituents of all forms of the biological system the exact conformation and three dimensional folding are highly connected to the protein functions, the restore of nativity of protein is crucial. Thus, critical evaluation of PC content serves as biomarkers of protein oxidative damage in various conditions like diabetes, ageing, neurodegeneration, smoking, chronic inflammatory diseases etc [8].

Current prospectives is wholly consistent with the hypothesis that there is enhanced ROS mediated damage to tissues in the most advanced states of periodontal disease, unless abated through antioxidant action.[25]

The assessment of PCs offers some advantages because it is a marker that occurs in the early stages of pathology and remains in circulation for a long time, compared to other biomarkers of oxidative stress (as malondialdehyde or 4-hydroxy-2-nonenal or glutathione). Their chemical stability (for 3 months at - 800C during storage) in different types of biological samples and its clinical accessibility makes PCs suitable for routine laboratory measurement [21].

At the same time, PCs level fulfill the four conditions of the ideal biomarker of oxidative stress: (1) accurately indicates the level of the oxidative damage, (2) it is an early indicator of the pathological process (3) it gives significant information of the pharmacologic response to a therapeutic intervention and (4) it evaluates the efficacy of antioxidants. [42]

In the present study we included four groups viz healthy, CP, CPS and CPST. these groups helped us to evaluate the role of PC in periodontal health and disease superimposed with risk factors like smoking and ST chewing.

The results obtained in the current study indicate (i) significantly higher levels of protein carbonyls in diseased whole unstimulated saliva in CPS and CPST in comparison to the other two groups, and (ii) a positive correlation with statistically significant difference in salivary PC among the four study groups.

When the values were plotted on a graph(I) we could find the gradual increase in PC levels from group 1 to groups 3 and marginally reduced in group 4, suggesting that the oxidative stress increases as the periodontal disease advances from heatlh to periodontitis with maximum collateral damage to proteins in group 3 as compared with group 4.

Graph (I): PC levels in Saliva

In the current study, we used saliva as the diagnostic tool because obtaining saliva can be low cost, noninvasive, simple and does not cause patient discomfort, it is a highly desirable body fluid for biomarker development for clinical application as it contains biomolecules such as DNA, mRNA, microRNA, protein, metabolites and microbiota [22].

A previous study has shown diurnal variations in salivary PC levels and therefore all salivary samples were obtained between 09:00 am and 12:00 pm to minimize temporal fluctuation in salivary redox homeostasis [38].

The results of our study are also in accordance with those [35]*which* reported an increase in PC levels in both serum and Gingival Crevicular Fluid of patients with CP as compared to healthy control. In a similar study done [40] in which PC levels in Gingival Crevicular Fluid where estimated to be highest in CP, as compared with healthy and gingivitis.

CS contains over 4000 different chemicals, 400 of which are proven carcinogens. CS also contains oxidants such as oxygen free radicals and volatile aldehydes , which are probably the major causes of damage to biomolecules exposed to CS, [16,5].

The findings of the present study are consistent with the previous work done [23,28] who showed in their research that there is significant increase in PCs in saliva following exposure to CS.

ST use has been associated with several oral manifestations localized at the site of ST placement. These manifestations include mucosal lesions and gingival-periodontal effects, such as gingival recession,gingival inflammation, changes in gingival blood flow and interproximal periodontal attachment loss[10,30].With due emphasis on this, we estimated the PC levels in subjects with smokeless tobacco chewing habit. It has been noted in our study for the first time that the levels of Protein carbonyls were significantly higher in CPS compared to healthy and CP subjects and CPST as well, Where as CPST values were found to be marginally lower than in CPS. As this was the first study assessing the levels of protein carbonyls among smokers and ST chewers with CP, a direct comparison with other research was not possible.

The increased protein carbonylation in smoking can be attributed to the effects of aldehydes present in the gas phase of cigarette smoke11,while placement of ST increases inflammatory mediators such as prostaglandin E2 (PGE2) and interleukin–1 (IL-1) in these developing sites[17]

There is no concrete data in literature signifying the extent of protein carbonylation in smokers and ST chewers with CP. We wanted to see if this aspect was correlated clinicobiochemicaly to the same as determined by the levels of protein carbonyls. This intriguing influence of periodontitis and smoking and ST on protein carbonylation warrants further exploration to determine the extent of cellular damage on

periodontium as we could reach a formal conclusion on the basis of these findings.

There were a few limitations of our study. The cross-sectional nature of the study design does not imply the temporal direction of the relationship between sST chewing and periodontitis. A larger sample would have helped us to achieve a more homogenous CPS and CPST population with respect to the duration of the habit. Further, the healthy controls were younger than the subjects in the diseased groups. Gender distribution was not possible as there was a vast difference in prevalence of smoking and ST chewing habit in female patients as compared to males in Indian population. Within the limitation of the study, it can be concluded that Smoking and ST chewing has an impact on periodontium as determined by the levels of PCs.

CONCLUSION

PCs constitute an important biomarker used in the early diagnosis of pathologies associated with an overproduction of ROS; Comprehensive evaluation of the current state-of-the-art concerning PC evaluation revealed that considerable progress has been made in different disease entities , and the development of new methods and techniques and on their applicability on different biological samples. Current study used the most valid method in determining PCs, however elucidation of the molecular identity of PCs and investigation of which amino acid residues suffered the carbonilation process is still a challenge. Given the overwhelming evidence of the negative impact of tobacco on periodontal tissues it is encouraging to note that such clinicobiochemical studies demonstrate newer diagnostic markers for better treatment planning under influence of risk factors associated with the periodontitis.

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ABBREVIATIONS

Protein Carbonyl (PC), Chronic periodontitis (CP), Smokers with Chronic Periodontitis (CPS), Smokeless Tobacco Chewers with Chronic Periodontitis (CPST), Cigarette Smoke (CS), Reactive Oxygen Species (ROS).

Conflict of interest: The authors have no conflict of interest to declare.

Table 1: Descriptive statistics of study population (mean±SD)

Study groups	Group 1	Group 2	Group 3	Group 4
Age	21.80 +2.89	42.10+10.51	43.03+9.86	36.07+9.13
Sex	17/13	15/15	30/00	25/05
GI	0.94+0.52	1.93+0.52	1.40+0.56	1.3+0.53
PPD	1.63+0.76	5.30+0.65	5.40+0.56	5.20+0.41
CAL	0.0	6.07+0.61	5.90+0.84	6.03+0.76

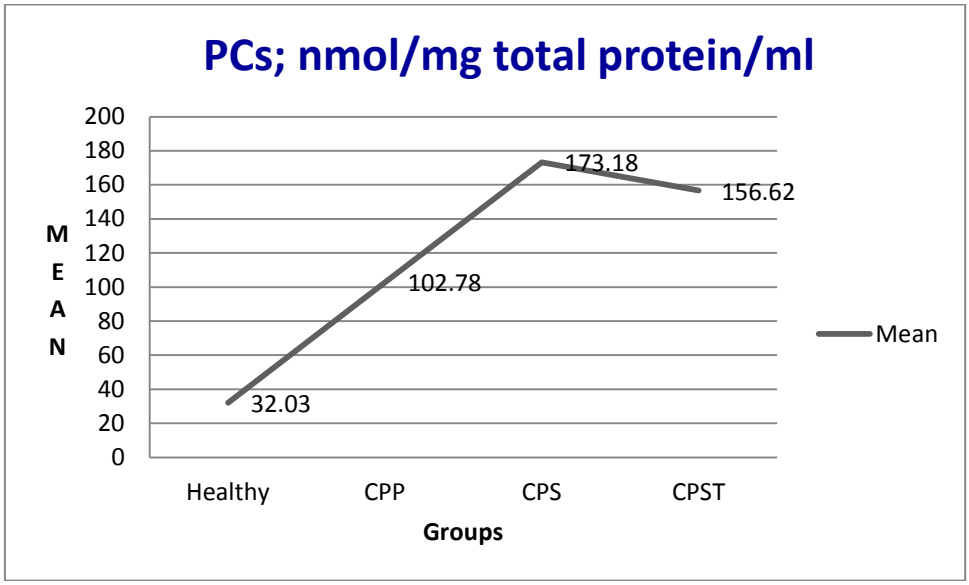
Table 2: Results of analysis of variance and pair-wise comparison using Tukeys multiple post hoc test of the mean salivary protein carbonyl levels between the four groups

Group	Mean	Standard Deviation	Standard Error
Healthy	32.03	7.14	1.30
CPP	102.78	18.17	3.32
CPS	173.18	29.68	5.42
CPST	156.62	31.99	5.84
F-value	212.5871		
p-value	0.00001*		
Pair wise comparisons of three groups by Tukeys multiple posthoc procedure			
Healthy vs CPP	p=0.0001*		
Healthy vs CPS	p=0.0001*		
Healthy vs CPST	p=0.0001*		
CPP vs CPS	p=0.0001*		
CPP vs CPST	p=0.0001*		
CPS vs CPST	p=0.0410*		

Table 3: Relationship of salivary protein carbonyl levels to clinical parameters

Parameters	Group 1	Group 2	Group 3	Group 4
GI	0.012	1.2540	0.7611	2.0653*
PPD	0.4833	0.6465	0.7094	0.6671
CAL	--	0.4263	0.6425	0.5904

P<0.05
*Significant at *P*<0.05, GI: Gingival Index; PPD: Probing Pocket Depth; CAL: Clinical Attachment Loss



Graph. (I): PC levels in Saliva

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