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Estimation of Salivary Malondialdehyde Levels in Smokeless Tobacco Chewers with Chronic Periodontitis-A Cross Sectional Clinico Biochemical Study

Estimación de los niveles de malondialdehído salival en masticadores de tabaco sin humo con periodontitis crónica: un estudio bioquímico clínico transversal

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ABSTRACT: Despite the reported effects of smokeless tobacco (ST) on the periodontium and high prevalence of ST use in rural populations and in males studies on this specific topic are limited. The purpose of this cross-sectional investigation was to measure lipid peroxidation (as an end product of oxidative stress) end product i.e. Malondialdehyde (MDA) in saliva of patients with gingivitis, chronic periodontitis and to assess the influence of smokeless tobacco on Salivary Malondialdehyde (S-MDA). Total 30 patients with gingivitis, 30 with chronic periodontitis and 30 Smokeless Tobacco Chewers with Chronic Periodontitis and 30 periodontally healthy subjects were included in the study. Plaque Index (PI), Gingival Index (GI), Probing Pocket Depth (PD), and Clinical Attachment Loss (CAL) were recorded followed by stimulated Saliva sample collection. Salivary MDA Levels were assessed by UV Spectrophotometry. There was a statistically significant increase in the salivary MDA levels in gingivitis, chronic periodontitis and in smokeless tobacco chewers with chronic periodontitis when compared with healthy group. Higher salivary MDA levels in gingivitis group,

chronic periodontitis, and smokeless tobacco chewers with chronic periodontitis reflects increasedoxygen radical activity during periodontal inflammation.

KEYWORDS: Periodontitis (CP); Smokeless tobacco chewer with chronic periodontitis (STCCP); Lipid peroxidation (LPO); Malondialdehyde (MDA); Oxidative stress (OS); Thio barbituric acid reacting substances (TBARS).

RESUMEN: A pesar de los efectos reportados del tabaco sin humo (TS) sobre el periodonto y la alta prevalencia del uso de TS en poblaciones rurales y en hombres, los estudios sobre este tema específico son limitados. El propósito de esta investigación transversal fue medir el producto final de la peroxidación lipídica (como producto final del estrés oxidativo), es decir, malondialdehído (MDA) en la saliva de pacientes con gingivitis, periodontitis crónica y evaluar la influencia del tabaco sin humo en el malondialdehído salival (S-MDA). Se incluyeron en el estudio un total de 30 pacientes con gingivitis, 30 con periodontitis crónica y 30 masticadores de tabaco sin humo con periodontitis crónica y 30 sujetos periodontalmente sanos. Se registraron el índice de placa (PI), el índice gingival (GI), la profundidad de la bolsa de sondeo (PD) y la pérdida de adherencia clínica (CAL), seguidos de la recogida de muestras de saliva estimuladas. Los niveles de MDA en saliva se evaluaron mediante espectrofotometría UV. Hubo un aumento estadísticamente significativo en los niveles de MDA en saliva en gingivitis, periodontitis crónica y en masticadores de tabaco sin humo con periodontitis crónica en comparación con el grupo sano. Los niveles más altos de MDA en saliva en el grupo de gingivitis, periodontitis crónica y masticadores de tabaco sin humo con periodontitis crónica reflejan un aumento de la actividad de los radicales de oxígeno durante la inflamación periodontal.

PALABRAS CLAVE: Periodontitis (CP); Masticador de tabaco sin humo con periodontitis crónica (STCCP); Peroxidación de lípidos (LPO); Malondialdehído (MDA); Estrés oxidativo (OS); Sustancias que reaccionan al ácido tio barbitúrico (TBARS).

INTRODUCTION

Periodontal diseases result from the complex interaction between pathogenic bacteria & the host immune inflammatory responses (1). Pathogens such as gram negative species, motile rods & spirochetes may have the ability to invade gingival tissues. The interaction between pathogenic bacteria & the host immune response is accompanied by an increase in cytokine expression & immunological activity in gingival tissues (2). The polymorpho nuclear leucocytes (PMNs) constitute the first line of defence against bacteria in the gingival sulcus (3). The antibacterial activity of PMNs & monocytes include oxygen dependent &oxygen independent mechanisms. The oxygen dependent pathway involves the production of reactive oxygen species (4).

ROS include O_2 derived free radicals, such as superoxide (O_2), hydroxyl (OH), nitric oxide (NO), Hydrogen Peroxide (H_2O_2) and hypochlorous acid (HOCL) (5,6). ROS are highly toxic, not only to the internalized microbial agent, but also the

extra cellular structure (6). Antioxidants, many of which are released locally at sites of inflammation by PMNs & other cells c provide protection against ROS. The balance is maintained by interaction of oxidants & antioxidants (7,8). Oxidative stress (OS) occurring as a consequence of imbalance between the formation of free O_2 radicals & interaction of these species by antioxidant defence system, is capable of causing damage to various cellular and extracellular constituents (9). ROS can cause damage via multiple mechanisms, e.g DNA damage, lipid peroxidation, protein damage, enzyme oxidation & the stimulation of pro-inflammatory cytokines release by monocytes & macrophages (10). Poly unsaturated fatty acids are highly susceptible to attack by ROS (ll) leading to lipid peroxidation, which has been shown to cause profound alteration in the structural integrity & function of cell membranes (11). Lipid peroxidation can be quantified by using the thiobarbituric acid reacting substances method (TBARS), which evaluates oxidative stress by assaying MDA the final product of lipid breakdown caused by oxidative stress (12). The most commonly used test for measurement of MDA is thiobarbituric acid reactive substances (TBARS).Cigarette smoking and tobacco chewing is considered to be most important environmental risk factors of periodontitis.

Cigarette smoke contains a large amount of oxidative species, & therefore represents a significant source of oxidative stress (13). Smokers demonstrate 2.6-6 times increased prevalence of periodontal diseases compared to non smokers (14). Another most, common form of tobacco consumption in the southern Asia, especially in India is in the form of Gutkha. Gutkha is composed of crushed areca nut, tobacco, catechu, paraffin, slaked lime & sweet or savory flavorings (15). Henceforth the term smokeless tobacco is used instead of gutkha in this study.

This form of tobacco has many oral effects, including leukoplakia, oral cancer and loss of periodontal support (recession), alveolar bone loss and staining of teeth and composite restoration (16). The chemical carcinogens in smokeless tobacco include polynuclear aromatic hydrocarbons (usually benzo[a]pyrene), polonium 210, and N-nitrosamines. Other chemicals include radium-226 and lead-210 (17). The main aim of the present study is to estimate the S-MDA levels in different groups with gingivitis, chronic periodontitis & smokeless tobacco chewers with chronic periodontitis, and in periodontally healthy subjects, and to correlate the S- MDA levels among each group and also to correlate the clinical periodontal parameters with the S-MDA levels.

MATERIALS AND METHODS

Total 30 gingivitis patients, 30 Chronic periodontitis patients, 30 smokeless tobacco chewers with chronic periodontitis and 30 systemically healthy subjects were selected from the OPD, Department of Periodontics, PMNM Dental College and Hospital, Bagalkot, Karnataka, India. Written informed consent was obtained from all subjects and the study protocol was approved by the Ethical Committee.

All subjects were systemically healthy. Subjects were excluded from the study if they had taken a course of non steroidal anti inflammatory drugs or antimicrobial drugs within a 6 month period before the study began; were pregnant or lactating; had used mouth washes or vitamin supplements within the previous 3 months; had a history of current drug use; or had special dietary requirements.

The selection of patients was made according to the criteria proved by the 1999 International Workshop for the classification of periodontal diseases and conditions. 30 subjects with generailized chronic

periodontitis characterized by at least 30% teeth with probing pocket depth >5mm and CAL>4mm were recruited. Tobacco Chewing was determined by verbal questionnaire. Tobacco Chewers were enrolled if they regularly chew Smokeless Tobacco (GUTKHA)-at least 1 sachet daily for at least 12 months. Healthy control group had no attachment loss with the teeth having probing depth<3mm & no bleeding on probing. These individuals were systemically and periodontally healthy volunteers. Prior to saliva sample collection, Plaque Index (PI); Gingival Index (GI), Probing Pocket Depth (PPD), Clinical Attachment Level (CAL) were measured. PD and CAL measures were obtained using a william's periodontal probe. All clinical periodontal measurements were performed by the same examiner.

Stimulated saliva samples were collected in a quiet room between 8AM-12PM. Patients were instructed not to eat or drink anything at least 1 hr before the procedure. About 2m1 of saliva was collected in Eppendroff tubes before clinical measurements and centrifuged at 10000 rpm for 15min. The supernatant was removed and stored in small Eppendroff tubes at -80°C until analysis. Salivary MDA levels were measured by a method based on the reaction of MDA with ThioBarbituric Acid to produce a pink colored complex that can be determined Spectro-photometrically & the absorbance was determined at 532nm.

The level of MDA was assayed in the saliva of study subjects, as previously described (Stalnaya and Garishvili 1977). Briefly, 0.3ml of collection saliva was mixed with 3ml of 0.025 M Tris-HCL and 0.175 M Acetic Acid. Then, 2.5ml of diluted saliva was mixed with 1ml of 17% (w/v) TCA and centrifuged at 4000g for 10min. The precipitate was pelleted by centrifugation and the supernatant was reacted with 1ml of 0.8% (w/v) TBA in a boiling water bath for 10min. After cooling to room temperature, the absorption of the supernatant was recorded at 532nm using a spectrophotometer. Statistical analysis was done by Kruskal Wallis ANOVA and Wilcoxon matched pair test, Mann-Whitney U test, Tukeys multiple post hoc procedure by using SPSS software.

RESULTS

The mean Plaque Index Scores in healthy group, gingivitis group, chronic periodontitis and smokeless tobacco chewers are shown in Table 1 the mean gingival index scores in all the groups were shown in Table 2. The differences in the values of BOP and CAL among the three groups of patients were statistically significant (P<0.05). The observed values for the mean S-MDA levels in healthy group, gingivitis group, CP group and in STCCP group were 5.42, 6.63, 8.96, 8.51 respectively.

The concentration of LPO product (MDA level) in the saliva of study subjects is shown in Table 3. The comparisons of the salivary MDA levels among the three groups of patients and the healthy control subjects are shown in Table 3. Significant differences in the MDA levels of patients with gingivitis, CP and STCCP in comparison with those of healthy subjects were noted (P<0.05). The differences in the levels of MDA among the three groups of patients were statistically significant (P<0.05).The correlation between the salivary MDA levels and the clinical parameters among the three groups of patients are shown in Table 4. The salivary MDA levels were positively correlated with the three clinical parameters (P<0.05). Table 5 shows the correlation between PI and GI scores with salivary malondialdehyde levels umol/ml in all groups. There was a positive correlation between gingival index and SML in gingivitis and chronic periodontitis.

Table 1. Comparison of groups as well as baseline scores of PI by Kruskal Wallis ANOVA and Wilcoxon matched pairs test.

Groups	Baseline PI		
	Mean	SD	Median
Healthy	-	-	-
Gingivitis	1.02	0.42	0.915
Chronic Periodontitis (CP)	1.25	0.62	0.94
STCCP	1.51	0.55	1.59
H-value		8.2872	
P-value		0.0159*	
Pair wise comparison of groups by Mann-Whitney U test			
Gingivitis vs CP		P=0.3119	
Gingivitis vs STCCP		P=0.0028*	
CP vs STCCP		P=0.0956	

Table 2. Comparison of groups as well as baseline scores of GI by Kruskal Wallis ANOVA and Wilcoxon matched pairs test.

Groups	Baseline PI		
	Mean	SD	Median
Healthy	-	-	-
Gingivitis	1.69	0.49	1.69
Chronic Periodontitis (CP)	1.91	0.53	1.99
STCCP	1.22	0.60	1.1
H-value		15.13523	
P-value		0.0005	
Pair wise comparison of groups by Mann-Whitney U test			
Gingivitis vs CP		P=0.1822	
Gingivitis vs STCCP		P=0.0105*	
CP vs STCCP		P=0.0002*	

Table 3. Comparison of Chronic periodontitis (CP) and Smokeless tobacco chewers with chronic periodontitis (STCCP) with pocket depth (PD) and attachment loss (CAL) by unpaired t test.

Variables	Time	Group	Mean	SD	t-value	p-value
PD	Baseline	CP	5.95	0.63	-0.5632	0.5758
		STCCP	6.05	0.64		
CAL	Baseline	CP	6.25	0.75	-2.4850	0.0163*
		STCCP	6.81	0.86		

*p<0.05.

Table 4. Comparison of groups as well as baseline scores of Salivary Malondialdehyde levels (SML in umol/ml) by one way ANOVA and paired t test.

Groups	Baseline SML	
	Mean	SD
Healthy	5.42	1.55
Gingivitis	6.63	1.32
Chronic Periodontitis (CP)	8.96	2.59
STCCP	8.51	2.35
F-value	13.9980	
P-value	0.0000*	
Pair wise comparison of groups by Tukeys multiple post hoc procedure		
Healthy vs Gingivitis	P=0.2658	
Healthy vs CP	P=0.0001*	
Healthy vs STCCP	P=0.0002*	
Gingivitis vs CP	P=0.0018*	
Gingivitis vs STCCP	P=0.0191*	
CP vs STCCP	P=0.8625	

*p<0.05.
There was a statistically significant difference in the salivary malondialdehyde levels when compared between healthy and CP & Healthy vs STCCP & Gingivitis vs CP & Gingivitis vs STCCP.

Table 5. CCorrelation between PI and GI scores with salivary malondialdehyde levels (umol/ml in all groups.

Time Point	Variables	Gingivitis Group	Chronic Periodontitis Group	STCCP group
Baseline	PI with SML	r = 0.0156	r = 0.1790	r = 0.1159
	GI with SML	r = 0.4630*	r = 0.5935*	r = 0.2008
	PD with SML	-	r = 0.0750	r = 0.1255
	CAL with SML	-	r = 0.1998	r = 0.2211

*p<0.05.
r-values are negative, means that, there is a negative correlation between them.
r-values are positive, means that, there is a positive correlation between them.

DISCUSSION

It is widely accepted that the host response to subgingival bacteria plays a critical role in periodontal pathogenesis (18) and that pathogenic processes are modified by environmental and acquired risk factors (19). Several ROS and lipid peroxidation products are produced in physiological quantities in the human body, but an overproduction of ROS occurs at sites of chronic inflammation (20). In normal physiology, there is a dynamic equilibrium between ROS activity and antioxidant defence capacity. When there is an imbalance, caused by a reduction in antioxidant defence and or ROS production or activity, oxidative stress results (21). Our study focused on lipid peroxidation in patients with gingivitis, periodontitis and the effect of smokeless tobacco chewing (STC) on these parameters.

The findings of our study suggest that gingivitis, periodontitis and smokeless tobacco chewing lead to significant changes in lipid peroxidation (MDA) in saliva. The combination of periodontitis and smokeless tobacco tended to result in significant differences in these parameters compared to those recorded in periodontally healthy control subjects. Lipid peroxidation is one of the most important reactions of free radicals. Tissue destruction by oxidative stress can be measured by the final end products of lipid peroxidation, such as MDA, one of many aldehydes formed during lipid peroxidation (22).

The mean S-MDA levels in healthy group, gingivitis group, CP group and in STCCP group were 5.42, 6.63, 8.96, and 8.51 respectively. When compared between groups the S-MDA levels were statistically significant in healthy versus CP, healthy vs STCCP, gingivitis vs CP & gingivitis vs STCCP. The S-MDA levels were not statistically significant when compared between CP & Si CC Positive correlation was found between G1 and S-MDA levels in gingivitis and chronic periodontitis group (23). Our results showed higher MDA levels in patients with gingivitis, periodontitis compared to healthy control, and MDA concentrations in smokeless tobacco chewers with chronic periodontitis were significantly higher than those in non smokeless tobacco chewing controls ($p<0.05$). Tsai *et al.* (2005) reported that lipid peroxidation in GCF and saliva was higher in diseased sites than in healthy sites and concluded that the balance between oxidative stress and antioxidant levels failed in periodontitis, resulting in increased tissue damage by ROS (10).

Panjamurthy *et al.* (2005) reported that MDA levels in plasma, erythrocytes, erythrocyte membranes and gingival tissues were significantly higher in periodontitis patient than in healthy controls (24). Celec *et al.* (2005) demonstrated increased salivary MDA levels in patients with periodontitis and found no correlation between MDA concentration in saliva and serum. They suggested that local oxidative stress in periodontitis may lead to increased MDA level (25). In the present study could find positive correlation between gingival index and MDA levels only in patients with gingivitis and chronic periodontitis.

Akalin *et al.* (2007) has shown significantly high levels of MDA in the saliva of patients with periodontitis. Specifically their study indicated that LPO concentration was correlated with the gingival index, probing depth and attachment level (26). The findings of our study suggest that both periodontitis and smokeless tobacco chewing lead to significant changes in lipidperoxidation in saliva. The combination of periodontitis and smokeless tobacco chewing tended to result in significant differences in these parameters compared to those recorded in periodontally healthy subjects. To the best of our knowledge this is the first study conducted to show the effect of smokeless tobacco on S-MDA levels. Our study reported statistically significant increase in s-MDA levels in STCCP. S-MDA levels were more in STCCP than healthy group and gingivitis group.

CONCLUSION

Within the limitations of this study, we can conclude that patients who use smokeless tobacco and suffer from periodontitis demonstrate mare lipid peroxidation in saliva than healthy subjects. Smokeless tobacco chewers with chronic periodontitis demonstrated higher oxidative damage and lipid damage biomarkers in saliva than healthier subjects. We suggested in this study that higher salivary MDA levels seemed to reflect increased oxygen radical activity during periodontal inflammation. However increased oxidative stress in periodontal microenvironment may not directly effect on severity of destructive periodontal diseases. Further investigations are needed to clarify the role of oxidative stress in pathogenesis of destructive periodontal diseases. However, the present study suggests that the impact of quantified tobacco use on periodontitis in small population may be limited to the importance of S-MDA levels and, the cardinal role of oral hygiene as one of the etiology of periodontal disease should also be assessed. Nevertheless, both oral hygiene and the quantified tobacco use may be considered as risk indicators for periodontitis. Further intervention with expanded study sample is acceptable to be more specific towards the effect of smokeless tobacco over S-MD levels.

CONFLICT OF INTEREST

Nil.

COMPETING INTERESTS

Not applicable.

FUNDING STATEMENT

Not applicable.

The manuscript is new and not being considered elsewhere.

This study was conducted in compliance with the protocol; ethical approval was obtained from the ethical committee of PMNM Dental College and Hospital, Bagalkot, Karnataka, India. The manuscript has been approved by all authors.

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