Original Article

Comparative Evaluation of Saliva's Oxidant–Antioxidant Status in Patients with Different Clinicopathological Types of Oral Leukoplakia

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ABSTRACT

Objectives: Despite advancements in the management of oral cancer, the mortality rate associated with it still remains a matter of concern. Early identification and intervention of precursor lesions such as leukoplakia have always been emphasized, as this can drastically improve the scenario. The oxidative stress has been implicated in the pathogenesis of several diseases, including oral cancer. The aim of this study was to evaluate salivary oxidant and antioxidant levels in patients with different clinicopathological stages of oral leukoplakia. **Materials and Methods:** An analytical study with case–control study design was conducted. Forty newly diagnosed cases of oral leukoplakia were considered in the case group. The equal number of age- and gender-matched subjects was included in the control study group. Unstimulated whole-saliva supernatant was used to determine the levels of lipid peroxidation, glutathione S-transferase, nitrites, and uric acid using ultraviolet visible spectrophotometer. The statistical comparisons were performed by independent Student's unpaired *t* test and one-way analysis of variance with post hoc analysis. Correlation analysis was performed among salivary parameters and with baseline variables. **Results:** End products of free radical damage and nitrite levels were significantly increased in patients with oral leukoplakia compared to controls. Conversely, levels of glutathione S-transferase and uric acid were significantly decreased in the study group in comparison with healthy subjects. Similar trends were seen along the clinical stages and histopathological grades of leukoplakia. **Conclusion:** Elevated levels of reactive species with a concomitant reduction in antioxidants in leukoplakia indicate its potential as an early diagnostic marker.

Keywords: *Free radicals, glutathione S-transferase, oral leukoplakia*

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Introduction

Deep concerns over the high mortality and morbidity rates of oral cancer still exist. Along with pharyngeal cancer, oral cancer is considered to be the ninth most common cause of malignancy across the globe[1] and the third leading cause of mortality in the developing countries.[2]

One of the significant causes for the high mortality rate is the delay in diagnosis of the potentially malignant disorders, the precursors of oral cancer.[3] Leukoplakia is widely documented as the most prevalent lesion in this category. Lack of awareness and inconspicuous nature of lesion are few among the various reasons documented for the delay in the diagnosis of these lesions. Although there are various modalities available for the early detection, biopsy still remains the gold standard. Incision biopsy is an invasive procedure, and so it adds to the list of reasons in the diagnostic delay.[4] Hence, there has been a compelling need for early diagnostic markers and noninvasive approach.

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Under physiological circumstances, there exists an oxidant–antioxidant balance. It gets disturbed under the influence of altered body states such as stress, exercise, hormonal imbalance, cardiovascular disease, neurodegenerative diseases, and type 2 diabetes.[5-7] This imbalance may induce excessive generation free radicals beyond the physiological limit, creating phenomenon known as oxidative stress (OS). This will bring about deleterious effects to the fundamental unit of body — the cell — where lipids, nucleic acids, and proteins being the major targets.[8] The oxidative damage caused by free radicals—reactive oxygen species (ROS) and reactive nitrogen species (RNS)—on lipids present in cell membranes is known as lipid peroxidation. Once initiated, it has a provocative nature, and thus produces more free radicals. The by-products of this process, for example, alkanes, malondialdehyde, and isoprostanes, have been routinely used as markers of lipid peroxidation assay and have been quantified to make an estimate for the damage.[9]

To counteract the actions of free radicals, the body has antioxidants present in all body fluids and tissues. Depending on the need and situation, they act accordingly, either by preventing the production of free radicals or by scavenging. Glutathione system of enzymes which includes glutathione, glutathione reductase (GSH), glutathione peroxidase (GPx), and glutathione S-transferase (GST) is one of the important groups of enzymes involved in the prevention of free radical generation. On the other hand, uric acid (UA) is a nonenzymatic antioxidant. Though it is plasma born, it accounts for the most abundant antioxidant present in saliva.[10]

OS has been found to be associated with oral diseases such as chronic periodontitis[11] and oral lichen planus.[12] Oral cancer is no exception, as various studies have proposed its involvement in carcinogenesis, playing a crucial role in initiation and progression by causing mutations in deoxyribonucleic acid (DNA) strands.[13]

Various studies in the past have successfully explored the markers of OS in plasma and tissue samples of oral premalignancy[14] and malignancy.[15] The results have shown raised levels of thiobarbituric acid reactive substances (TBARS) and depleted levels of antioxidant enzymes in patients with oral cancer when compared with those with leukoplakia and control subjects. An interesting observation was made with respect to the pattern of antioxidant enzymes in tissue and plasma compartments. The diseases tissue showed raised levels of GSH and GPx, presumably giving a selective growth advantage.[16,17]

In search of a noninvasive approach and to study the local environment, saliva has been a topic of research from the past few years. Few studies with limited parameters of oxidative[18] or nitrative[19] stress have evaluated in saliva.

This study outscores the previous investigators in terms of comparing parameters indicative of oxidative and nitrative stress in various clinical stages and dysplastic grades of leukoplakia. This, in turn, will reflect their impact on malignant transformation. This study also attempts to study the correlation of salivary parameters with baseline variables related to habit and age.

The aim of the study was to evaluate the salivary lipid peroxidation and antioxidants, namely GST and UA, in patients with various histopathological and clinical grades of leukoplakia. This study focused on the importance of various antioxidant enzymes and free radical damage in saliva to reestablish its role in the pathogenesis of oral premalignancy. Estimating these levels could serve as a viable marker to prevent the disease in its early stage, thereby avoiding progression to malignancy and having a better prognosis of the condition.

Materials and Methods

A prospective, case–control study design was adopted for this study. It included a total of random 80 subjects, which were divided into two study groups. Forty patients with clinically and histopathologically proven new cases of oral leukoplakia (OLEP) were considered in the case group (study group B). An equal number (40) of age- and sex-matched, healthy subjects were included in the control group (study group A). Patients who had any concomitant disease, such as diabetes, hypertension, and liver or kidney disorders, or other systemic diseases; those having lesion other than leukoplakia; and those with leukoplakia, who had previously undergone any treatment, were excluded from the study.

All the subjects for the study were taken from the outpatient unit of Department of Oral Medicine, Diagnosis, and Radiology. Before the inclusion of subjects into the study group, they were subjected to complete intraoral examination with emphasis on the lesion, so as to make clinical staging as per the OLEP criteria. In addition to this, a detailed history of tobacco usage (form, duration, and frequency) was recorded in the interview section of case documentation, apart from the demographic data. For the purpose of diagnosis and establishing the grading of lesion, an incision biopsy was performed.

The local institutional ethical committee approval was obtained before the commencement of the study (AU/19.11.2010). Written informed consent was obtained from all the participants of the study.

Sample collection

Unstimulated whole saliva was collected between 8 AM and 11 AM. The subjects were instructed to spit into a sterile universal plastic container for 10 minutes, not forcibly to avoid blood contamination. Two milliliters of saliva was collected and transferred for biochemical analysis.

Salivary analysis

The salivary samples were centrifuged at 800*g* at 4°C for 10 minutes. The supernatant was taken for biochemical analysis:

1. Lipid peroxidation (TBARS): Lipid peroxides in saliva were assayed by the method of Ohkawa *et al.*[20] The color formation with thiobarbituric acid was used as the index. Values are expressed as *n* moles/mL saliva.
2. GST: An enzyme immunoassay was used allowing the quantitative determination of the human GST using method of Habig and Jakoby.[21] The amount of converted substrate, indirectly proportional to the amount of GST antigen in the sample, was photometrically determined at 450 nm. Values are expressed as ng/mL saliva.
3. Nitrites: Nitric oxide (NO) was measured in terms of its products, nitrite (NO_2) and nitrate (NO_3), by the Griess method.[22] Values are expressed as µmol/L saliva.
4. Uric acid: UA concentration was measured using method of Bablok *et al.*[23] In the assay, UA is transformed by uricase into allantoin and hydrogen peroxide, which, under the catalytic influence of peroxidase, oxidizes the chromogen to form a red compound. It is read at a wavelength of 546 nm and values are expressed as mg/mL saliva.

Statistical analysis

Regarding sample size, post hoc analysis was performed using software—G Power 3.1.9.2 (Heinrich-Heine-Universität Düsseldorf, Germany)—at confidence interval (α) of 0.05, effect size of 0.8, and two-tail test. The sample size achieved a statistical power of 0.94. All quantitative and qualitative data were expressed in mean ± SD (standard deviation) and number/percentages, respectively. All the variables of the study were statistically analyzed for the mean values, SD, and *P* value. The intergroup comparison of salivary parameters were performed by independent *t* test. The comparative analysis was also performed between the clinical stages and histopathological grades by analysis of variance followed by post hoc Tukey test. The causal analysis was carried out by Pearson and Spearman correlation, depending on the nature of the variable. The data were analyzed using SPSS, version 21.0, package (SPSS, Chicago, IL). In all the aforementioned tests, the *P* value of <0.05 was considered statistically significant.

Results

This study was composed of two study groups with a sample size of 40 subjects in each group. The biographic data of the case group showed a mean age of 45.20 ± 11.009 years and male predominance (75%). Majority of the patients had leukoplakia lesion on buccal mucosa (75%), and alveolus was found to have the least number (5%) of cases. Lateral border of tongue and vestibule contributed equally (10%). We found that all our patients used tobacco with or without additives with an average duration of habit as 20.80 ± 10.469 years and average frequency of 7.85 ± 3.317 times per day [Table 1].

Salivary parameters indicative of OS, namely TBARS and nitrates, showed a significant ($P > 0.001$) increase in patients with leukoplakia when compared with the control group. On the contrary, the antioxidants, GST and UA, showed a significant ($P < 0.001$) decrease in the case group [Table 2].

Considering the objective of this study, the pattern of salivary parameters was assessed in the various clinical stages and histopathological grades of leukoplakia. The TBARS and nitrate levels showed a significant ($P > 0.001$) increasing trend along the stages and grades. Unlike previously mentioned parameters, GST and UA levels showed a significant ($P > 0.001$) decreasing trend along the clinical stages and histopathological grades [Tables 3 and 4].

Post hoc analysis was performed to explore the difference between the individual stages and grades. All clinical stages when compared in all possible combination of pairs were found to have significant ($P > 0.001$) increase in nitrate levels in the relatively advanced clinical stage in the pair under evaluation. Although TBARS was found to be significantly increased, the significance level varied in clinical stages. Clinical stage IV was found to be consistently very highly significant ($P > 0.001$) in comparison with other stages [Table 3].

The analysis was also carried out among various histopathological grades using Tukey post hoc test. Where nitrates showed a significant ($P < 0.01$) increase in all advancing grades when compared with mild ones, TBARS showed similar result only with moderate dysplasia compared with severe dysplasia [Table 4]. A very high significant decrease ($P > 0.001$) was observed in GST and UA levels in comparatively severe grade

Table 1: Descriptive analysis for sample size and variables related to biographic data, tobacco habit, site of lesion, and clinical staging and histopathological grading in different study groups

S. No.	Variable	Study group	Frequency	Total		
1	Sample size (*n*)	Group A (control)	40	80		
		Group B (leukoplakia)	40			
	Variables related to biographic data					
2	Age, expressed as mean ± SD	Group A		39.55 ± 9.106		
		Group B		45.20 ± 11.009		
3	Gender, expressed as number (%)			Responses		
			Male	Female		
		Group A	30 (75)	10 (25)		
		Group B	30 (75)	10 (25)		
	Variables related to tobacco habit					
4	Duration of habit (years), expressed as mean ± SD	Group B		20.80 ± 10.469		
5	Frequency of habit (no. of times/day), expressed as mean ± SD	Group B		7.85 ± 3.317		
	Variables related to site of lesion					
6	Site of lesion, expressed as number (%)	Group B		Responses		
			Buccal mucosa	Alveolus	Vestibule	Tongue
			30 (75)	2 (5)	4 (10)	4 (10)
	Variables related to clinical stages and histopathological grades of group B—leukoplakia					
7	Clinical staging (OLEP stages), expressed as number (%)	Group B		Responses		
			Stage I	Stage II	Stage III	Stage IV
			8 (20)	14 (35)	12 (30)	6 (15)
8	Histopathological grading—dysplasia, expressed as number (%)	Group B		Responses		
			Mild	Moderate	Severe	
			10 (25)	14 (35)	16 (40)	

OLEP = oral leukoplakia, SD = standard deviation

Table 2: Intergroup comparative analysis of salivary parameters related to lipid peroxidation and antioxidant enzymes

S. No.	Variable	Study group A (mean ± SD)	Study group B (mean ± SD)	*P* value
1	TBARS	3.398 ± 0.017	3.760 ± 0.012	0.000‡
2	Nitrates	74.950 ± 7.702	174 ± 8.834	0.000‡
3	GST	226.850 ± 4.520	187.150 ± 4.709	0.000‡
4	Uric acid	3.667 ± 0.355	2.174 ± 0.261	0.000‡

Independent Student's *t* test (test of significance) is applied at 95% confidence interval. TBARS = thiobarbituric acid reactive substances, GST = glutathione S-transferase, SD = standard deviation

‡*P* value < 0.001.

between the grades chosen for comparison. A consistent finding was observed in all possible combinations of mild, moderate, and severe dysplasia [Table 4].

Analysis was carried to evaluate the causal association among the variables. In respect to salivary parameters, the pair of TBARS–nitrates and GST–UA showed a significant ($P > 0.001$) positive correlation with each other. However, TBARS–nitrates showed significant negative correlation with GST–UA. Among the variables related to habit, duration of adverse habit showed individual significant ($P < 0.05$) positive correlation with TBARS and nitrates. Contrary (significant negative correlation) results ($P < 0.05$) were seen with UA. A significant positive ($P > 0.001$) correlation was observed between the lipid peroxidation parameters (TBARS and nitrates) with clinical stages and histopathological grades. The parameters of antioxidants (GST and UA) displayed to have a significant negative correlation ($P > 0.001$) with the stages and grades [Table 5].

Discussion

Tobacco is a well-known risk factor for oral cancer. The tobacco-specific nitrosamines act as an exogenous source of ROS and RNS. These highly unstable chemical entities, when present in abundance and coupled with the depletion of antioxidant enzymes, lead to the development of OS. Lipid peroxidation, protein, and

Table 3: Comparative analysis of salivary parameters related to lipid peroxidation and antioxidant enzymes within clinical stages (OLEP stages) of study group B

S. No	Variable	Study group B—clinical stages				*P* value
		Stage I (Mean ± SD)	Stage II (Mean ± SD)	Stage III (Mean ± SD)	Stage IV (Mean ± SD)	
1	TBARS	3.747 ± 0.004 b†c‡d‡	3.755 ± 0.002 a†c*d‡	3.761 ± 0.006 a‡b*d‡	3.786 ± 0.004 a‡b‡c‡	**0.000**‡
2	Nitrates	162.250 ± 0.886 b‡c‡d‡	170.143 ± 2.315 a‡c‡d‡	179 ± 2.256 a‡b‡d‡	188.667 ± 1.366 a‡b‡c‡	**0.000**‡
3	GST	194.250 ± 0.886 b‡c‡d‡	188.714 ± 1.069 a‡c‡d‡	184 ± 0.852 a‡b‡d‡	180.333 ± 0.5164 a‡b‡c‡	**0.000**‡
4	Uric acid	2.570 ± 0.103 b‡c‡d‡	2.221 ± 0.140 a‡c‡d‡	2.031 ± 0.060 a‡b‡d†	1.823 ± 0.080 a‡b‡c†	**0.000**‡

One-way ANOVA with post hoc Tukey test (test of significance) is applied at 95% confidence interval. a = compared to stage I, b = compared to stage II, c = compared to stage III, d = compared to stage IV, TBARS = thiobarbituric acid reactive substances, GST = glutathione S-transferase, SD = standard deviation

*P value < 0.05; †P value < 0.01; ‡P value < 0.001.

Table 4: Comparative analysis of salivary parameters related to lipid peroxidation and antioxidant enzymes within histopathological grades of study group B

S. No.	Variable	Study group B—histopathological grades			*P* value
		Mild dysplasia (mean ± SD)	Moderate dysplasia (mean ± SD)	Severe dysplasia (mean ± SD)	
1	TBARS	3.749 ± 0.005 c‡	3.755 ± 0.004 c†	3.769 ± 0.014 a‡b†	0.000‡
2	Nitrates	163.800 ± 3.359 b†c‡	171.571 ± 4.255 a†c‡	182.500 ± 5.341 a‡b‡	0.000‡
3	GST	193.400 ± 1.955 b‡c‡	187.857 ± 1.875 a‡c‡	182.625 ± 1.995 a‡b‡	0.000‡
4	Uric acid	2.512 ± 0.152 b‡c‡	2.198 ± 0.141 a‡c‡	1.942 ± 0.112 a‡b‡	0.000‡

One-way ANOVA with post hoc Tukey test (test of significance) is applied at 95% confidence interval. a = compared to mild dysplasia; b = compared to moderate dysplasia; c = compared to severe dysplasia, TBARS = thiobarbituric acid reactive substances, GST = glutathione S-transferase, SD = standard deviation

†P value < 0.01; ‡P value < 0.001.

DNA damage are the various means by which cellular damage occurs during OS. This process can eventually lead to the initiation of carcinogenesis.[13]

Evaluation of ROS/OS

In this study, the end products of lipid peroxidation, TBARS, were found to be significantly ($P < 0.001$) raised in salivary samples of patients with leukoplakia, when compared to the controls [Table 2]. Similar results were documented in other potentially malignant disorder oral submucosa fibrosis.[19] We also found a positive correlation ($P < 0.05$) of TBARS with the frequency of tobacco chewing habit [Table 5].

Tobacco, when consumed in the form of smoke or smokeless, significantly alters the saliva. The chewable form exerts an additional chronic impact on the oral mucosa at the site of placement of tobacco. The local tissue absorbs high amounts of nicotine and its associated nitrosamines with genotoxic potential, leading to a state of chronic inflammation and eventually OS. The increasing burden of ROS and the mutagenic end product of lipid peroxidation transforms a normal cell into a mutated cell. The increasing frequency of tobacco exposure escalates the ROS and their mediated cellular damage, thus favoring the malignant transformation.[24]

Evaluation of RNS/nitrative stress

In this study, the significantly ($P < 0.001$) raised levels of salivary nitrates are observed in the case group [Table 2]. Our results are in agreement with previous studies.[19] The frequency of tobacco intake has shown a significant ($P < 0.05$) positive correlation [Table 5].

Human salivary gland tends to concentrate nitrates in high amounts and so is seen in saliva. Nitrates can undergo a series of chemical conversions to eventually produce carcinogenic nitrosamines (RNS) via formation of intermediates called nitrites. These species when in excess amount along with the deficiency of antioxidants develop nitrative stress.[19] Saliva of tobacco chewers modifies the oral environment, which might promote the conversion of nitrates into nitrites and NO in large amounts. They act in different ways to alter the redox metabolism, either by increasing the production of RNS or by scavenging the antioxidants.[25] As saliva maintains intimate contact with the site of lesion, probably nitrative stress plays a leading role in comparison to OS. On the basis of the results of this study, where we have found a strong positive correlation ($P < 0.001$) between the oxidative and nitrative stress markers, namely TBARS and nitrates [Table 5], it seems that they act synergistically in the initiation and progression of carcinogenesis.

Evaluation of antioxidant enzymes: UA and GST

We have observed significantly ($P < 0.001$) reduced levels of salivary antioxidants, GST and UA, in the study group when compared with the control group [Table 2]. Antioxidant enzymes, in general, are present

Table 5: Correlation analysis of salivary parameters with variables related to habit, biographic, clinical stages, and histopathological grades

S. N.	Variable	Salivary parameters				Habit related		Biographic related		Clinical and histopathology related		
		TBARS	Nitrates	GST	Uric acid	Duration of habit	Frequency of habit	Age	Gender	Site	Clinical stage	HP grade
1	TBARS	—	0.000‡ (0.848)	0.000‡ (−0.812)	0.000‡ (−0.719)	0.089 (−0.273)	0.024* (0.357)	0.307 (−0.166)	0.010* (0.401)	0.104 (0.261)	0.000‡ (0.848)	0.000‡ (0.709)
2	Nitrates	0.000‡ (0.848)	—	0.000‡ (−0.96)	0.000‡ (−0.87)	0.287 (−0.172)	0.042* (0.324)	0.672 (−0.069)	0.008† (0.411)	0.727 (0.057)	0.000‡ (0.961)	0.000‡ (0.864)
3	GST	0.000‡ (−0.812)	0.000‡ (−0.966)	—	0.000‡ (0.909)	0.516 (0.106)	0.054 (−0.307)	0.905 (−0.019)	0.007† (−0.422)	0.528 (−0.103)	0.000‡ (−0.963)	0.000‡ (−0.906)
4	Uric acid	0.000‡ (−0.719)	0.000‡ (−0.878)	0.000‡ (0.909)	—	0.810 (−0.039)	0.012* (−0.392)	0.560 (−0.095)	0.044* (−0.321)	0.776 (0.047)	0.000‡ (−0.899)	0.000‡ (−0.859)

Pearson and spearman correlation is applied at 95% confidence interval for quantitative (TBARS, nitrates, GST, uric acid, duration of habit, frequency of habit, and age) and qualitative (gender, site, clinical stages, and histopathological grades), respectively. TBARS = thiobarbituric acid reactive substances, GST = glutathione S-transferase, HP = histopathological

*P value < 0.05; †P value < 0.01; ‡P value < 0.001.

in the system to counteract the oxidants and so found to have a significant ($P < 0.001$) negative correlation in our study. At the same time, GST and UA are found to have significant ($P < 0.001$) positive correlation, restabilizing their synergistic action of defense [Table 5].

UA is a free radical–scavenging antioxidant, contributing to about 85% of total antioxidant capacity in saliva. It also acts as a substrate for GST in the detoxification of peroxides and other ROS. The depletion of these antioxidants complements the steep increase in ROS and RNS and thus explains the rationale of the initiation/progression of carcinogenesis. Similar results have been documented in the patients with oral cancers [25] and even in smokers in comparison to nonsmokers.[26] Unlike UA, glutathione (GSH) is one of the major antioxidants present in intracellular compartment. It assumes a crucial protective role for the cells from the toxic peroxides that have great potential in enhancing the load of reactive species. GSH brings down these actions by multiple mechanisms acting simultaneously. To mention few, it includes the detoxification of carcinogens, direct action of peroxides, and retarding the process of lipid peroxidation. It can be understood from this, that reduction in their levels in local environment will promote the initiation and progression of diseases such as OLEP.[13]

Comparative and correlation analysis of parameters with leukoplakia stages and grades

An interesting finding of the study was observed when all parameters were compared along the various clinical stages and grades of leukoplakia. Where oxidative/nitrative parameters (TBARS and nitrates) showed the significant increasing trend, the antioxidant parameters (GST and UA) displayed gradual decline [Tables 3 and 4]. On close observation, it was seen that nitrates were showing consistently high significant variation compared to TBARS. A strong positive correlation was also observed between the nitrates and stages/grades. Heavy amounts of nitrates in saliva and its proximity to the lesion are playing a pivotal role in the progression and malignant transformation of the lesion. With the pattern of correlation and trend of parameters seen along the stages and grades of leukoplakia, we can assume that the oxidative/nitrative stress is building up significantly with the progress of disease.

In the light of the results of this study, it is evident that the salivary analysis has found to be comparable to serum and tissue analysis in terms of these parameters. At the same time, ease, noninvasiveness, low cost, and unskilled procedure of collection make saliva outscore from their previously mentioned possible counterparts.

This study has used limited parameters from the elaborate list of oxidants and antioxidants involved in

the redox metabolism with complex web of interactions. The future studies should be undertaken with other members involved in redox metabolism to identify parameters with decisive role. This will pave the way to develop salivary biosensors, which can be used as point of care in the outpatient clinic for the early detection of potentially malignant disorders.

Conclusion

Strong correlations between elevated salivary TBARS and nitrite levels and with decreased antioxidant levels suggest that they have a leading role in the progression of potentially malignant conditions. Although saliva has emerged as a potential diagnostic fluid in the early detection of OLEP, more studies need to be carried out to evolve it into a sensitive and specific tool.

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Conflicts of interest

There are no conflicts of interest.

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