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Evaluation of salivary and serum lipid peroxidation, and glutathione in oral leukoplakia and oral squamous cell carcinoma

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Abstract: Lipid peroxidation induced by reactive oxygen species (ROS) is involved in the pathogenesis of malignancy. Overall, lipid peroxidation levels are indicated by malondialdehyde (MDA), which is the most frequently used biomarker to detect oxidative changes. Antioxidant defense systems such as glutathione (GSH) limit cell injury induced by ROS. Therefore, MDA and GSH can be used to monitor oxidative stress (OS). Hence, this study aimed to evaluate and compare both salivary and serum levels of MDA and GSH in oral leukoplakia and oral squamous cell carcinoma (OSCC) patients, and healthy controls. The study included 100 subjects comprising 30 apparently healthy controls, 30 patients with oral leukoplakia and 40 clinically and histologically diagnosed patients with OSCC. Saliva and blood samples were obtained and evaluated for MDA and GSH. The study revealed enhanced MDA levels in saliva and serum in oral leukoplakia and OSCC patients as compared to controls. On the other hand, significant decreases were seen in serum and salivary GSH levels in oral leukoplakia and OSCC patients as compared to controls. Augmentation of OS in blood and saliva is reflected by increase in MDA and decrease in GSH levels, indicating that tumor processes cause an imbalance of oxidant-antioxidant status in cell structures.

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Introduction

Oral squamous cell carcinoma (OSCC) is one of the ten most frequent cancers and accounts for 2-4% of all new cancers worldwide (1). Five-year mean survival rate remains very low, despite improvements in diagnostic and treatment modalities (2,3). Two-thirds of oral cancer patients, are diagnosed at advanced tumor stages, where survival drops to a little more than 30% and its prognosis is unpredictable (4,5). It is also the leading cancer site in India, and its high incidence reflects tobacco as a major etiological cause (6).

Tobacco is an exogenous source of reactive oxygen species (ROS) that subsequently leads to oxidative stress (OS). Tobacco products cause increase in free radicals and ROS production, which have a pathognomonic role in multistep carcinogenesis. They initiate mutagenic events by causing DNA damage that ultimately leads to degeneration of cellular components. Thus, free radicals and ROS stimulate malignant transformation and progression (7,8).

ROS and free radicals primarily target peroxidation of polyunsaturated fatty acids (PUFAs) in membrane lipids (9). They also interact with cellular DNA causing DNA damage and interference with its repair, which is a major cause of pathobiological changes in cancer cells. Peroxidation of membrane lipids generates well characterized, mutagenic lipid peroxidation end products, such as Lipid

hydroperoxides (LHP) and malondialdehyde (MDA) (10). MDA is a major genotoxic carbonyl compound generated by lipid peroxidation and during arachidonic acid metabolism for the synthesis of prostaglandins. Hence, MDA levels are used to indicate oxidative and cellular damage to tissues due to ROS and free radicals (9,11).

In normal cellular processes, cells are capable of neutralizing the deleterious effects of ROS and free radicals by several intracellular and extracellular anti-oxidative systems. Any change in one of these systems, breaks this equilibrium leading to OS, resulting in an overall increase in cellular levels of ROS that can initiate lipid peroxidation and induce oxidative DNA damage (12).

Glutathione (GSH) is the major intracellular anti-oxidant, and plays a critical role in protecting organisms against toxicity and disease by detoxifying deleterious hydrogen peroxide and alleviating OS caused by enhanced free radical production, as well as providing reducing capacity for several reactions (9,13). It is the major thiol compound that protects against oral cancer development as it detoxifies carcinogens and the effects of lipid peroxidation through Phase II conjugation, maintaining immune function by regulating lymphocytic proliferation and mitogenic responses (14).

The oxidant and anti-oxidant status of an individual plays an important role in the pathogenesis, development and progression of potentially malignant oral lesions (15). Therefore, the extent of OS in the body can be convincingly administered by evaluation of MDA and GSH, which were found to be reliable markers. Despite the high prevalence of pre-cancerous lesions in India and their potential to undergo malignant transformation (16), very few studies have evaluated serum and salivary MDA and GSH in oral pre-cancer patients. Hence, the present study was carried out to evaluate and compare serum and salivary MDA and GSH in oral leukoplakia and OSCC patients, and to establish the diagnostic efficacy of saliva in evaluating salivary levels of MDA and GSH in oral pre-cancer and OSCC patients.

Materials and Methods

Source of data

The study was independently reviewed and approved by Ethical Committee of Pacific Dental College and Hospital, Udaipur, Rajasthan, India (PDCH/12/PU-11-1176), and conforms to the provisions of the World Medical Association, Declaration of Helsinki. The study was conducted at the Outpatient Department of College, for a period of 6 months which included 100 subjects divided into three groups: Group I: 30 apparently healthy volunteers (controls), Group II: 30 clinically and histologically diagnosed patients with oral leukoplakia, Group III: 40 clinically and histologically diagnosed patients with OSCC, of which 20 each had well differentiated and moderately differentiated OSCC.

All individuals included in the study were aged from 30 to 80 years. Written informed consent was obtained from the patients, after the planned study was explained in detail. Patients with underlying systemic diseases, cancers other than oral cancer and previously treated cases for oral cancer were excluded from the study. A detailed history with thorough clinical examination was performed, and the findings were recorded. The clinical characteristics of controls, oral leukoplakia and oral cancer patients (age, distribution according to tobacco habits and site, clinical stage and histopathological grades) are shown in Table 1.

Sample collection

Saliva and blood samples were collected from each subject after overnight fasting. Five milliliters of venous blood was drawn from the selected patients using a sterile disposable syringe taking full precautions to prevent hemolysis. At the same time, whole unstimulated saliva samples were collected. Subjects were asked to rinse the mouth thoroughly, and were then directed to spit into a sterile plastic container.

MDA, the marker of lipid peroxidation was estimated as thiobarbituric acid-reactive substances (TBARS) (17). To 1 mL of sample, 1.5 mL of 0.8% thiobarbituric acid (TBA) was added. Then, 1.5 mL of acetic acid and 0.4 mL of 8.1% sodium dodecyl sulfate were added. Distilled water was added to make the mixture up to 5 mL, and it was then placed in a hot water bath at 95°C for 1 h. The mixture was allowed to cool, and 5 mL of pyridine and *n*-butanol (15:1, v/v) along with 1.0 mL of distilled water were added. The mixture was vortexed and centrifuged at 4,000 rpm for 10 min. With a spectrophotometer, absorbance of the upper layer was measured at 532 nm against distilled water. When allowed to react with TBA, MDA formed a colored complex that was measured using the spectrophotometer.

GSH was estimated using the method of Beutler et al., based on reduction of 5,5/-dithiobis-(2 nitrobenzoic acid) (DTNB) by GSH (18). The technique employs meta-phosphoric acid for protein precipitation, and the supernatant obtained on reaction with 5-5'dithiobis-2-nitrobenzoic acid resulted in a yellow colored derivative that was assayed with a spectrophotometer at 412 nm.

Table 1 Clinical and sociodemographic details of subjects

Characteristics	Control group (Group I)	Oral leukoplakia group (Group II)	Oral cancer group (Group III)
No. of patients	30	30	40
Mean age	48.3 years	51.7 years	68.4 years
Habits	None	Tobacco/quid chewers: 11 Tobacco chewers and smokers: 7 Tobacco and lime chewers: 7 Tobacco smokers: 5	Tobacco and lime chewers: 9 Tobacco chewers and smokers: 8 Tobacco chewers, smokers, alcoholics: 6 Betel nut chewer: 5 Tobacco, lime chewers and Betel nut chewer: 5 Betel leaf, betel nut, Tobacco and lime chewers: 7
Sites affected	N/A	Buccal mucosa: 12 Tongue: 5 Retromolar area: 5 Alveolus: 8	Anterior 2/3rd of the tongue: 8 Buccal mucosa: 10 Alveolus: 6 Soft palate: 3 Buccal and lingual vestibule: 4 Floor of mouth: 4 Palate: 5
Clinical diagnosis	N/A	Speckled leukoplakia: 9 Homogenous leukoplakia: 21	Stage II: 13 Stage III: 16 Stage IV: 11
Histopathological diagnosis	N/A	Mild dysplasia: 13 Moderate dysplasia: 9 Severe dysplasia: 8	Well differentiated OSCC: 20 Moderately differentiated OSCC: 20

Statistical analysis

Data were statistically analyzed by SPSS using unpaired *t*-test for significance of differences between each group. *P* values of less than 0.05 were considered to be statistically significant. The normality of data was checked before the statistical analysis was performed using SPSS. Shapiro-Wilk's test ($P > 0.05$) and visual inspection of histograms showed that values were normally distributed.

Results

Mean and standard deviation values for serum MDA levels in oral leukoplakia patients were greater than in controls ($P < 0.05$). Oral cancer patients had the highest serum MDA levels 6.02 ± 0.43 nmol/mL ($P < 0.001$) as compared with controls and oral leukoplakia patients (Table 2). However, the difference was not significant ($P > 0.05$) when the mean values were compared between well and moderately differentiated OSCC patients (Table 3).

The markers revealed a similar trend in saliva. Comparative assessment of salivary MDA levels between controls (19.98 ± 0.81 nmol/dL) and oral leukoplakia patients (20.87 ± 1.23 nmol/dL) depicts a significant difference ($P < 0.05$) while the difference was highly significant ($P < 0.001$) when salivary MDA levels were compared between oral leukoplakia and oral cancer patients (32.75 ± 3.03 nmol/dL) (Table 2). However, the difference was not significant ($P > 0.05$) when the values of well and moderately differentiated OSCC patients were compared (Table 3).

On the other hand, highly significant ($P < 0.001$) decreases were observed in serum GSH levels when the control group (32.18 ± 5.53 µmol/dL) was compared with the oral leukoplakia group (21.47 ± 3.35 µmol/dL) and the OSCC group (17.31 ± 1.55 µmol/dL) (Table 2). However, the results were not significant ($P > 0.05$) when mean values of well and moderately differentiated OSCC patients were compared (Table 3).

Similarly, in the case of salivary GSH, marker levels revealed a significant decrease ($P < 0.001$) when mean values in the control group (9.74 ± 0.53 µmol/dL) were compared with the oral leukoplakia (8.67 ± 1.2 µmol/dL) and OSCC groups (7.04 ± 0.67 µmol/dL) (Table 2), but the change was not significant ($P > 0.05$) when well and moderately differentiated OSCC patients were compared (Table 3).

In the present study, serum and salivary mean MDA and GSH levels in relation with histopathological grades in oral leukoplakia patients were recorded. Based on the statistical analysis, it was observed that the difference in levels of MDA and GSH between different histopathological grades of oral leukoplakia patients was not

Table 2 Evaluation and comparison of mean ± SD values for salivary and serum MDA and GSH in controls, and oral leukoplakia and OSCC patients

Groups	MDA		GSH	
	Saliva (nmol/dL)	Serum (nmol/mL)	Saliva (nmol/dL)	Serum (nmol/dL)
Control	19.98 ± 0.81	2.93 ± 0.79	9.74 ± 0.53	32.18 ± 5.53
Oral leukoplakia	20.87 ± 1.23	3.31 ± 0.41	8.67 ± 1.20	21.47 ± 3.35
	$P < 0.05$	$P < 0.05$	$P < 0.001$	$P < 0.001$
Oral leukoplakia	20.87 ± 1.23	3.31 ± 0.41	8.67 ± 1.20	21.47 ± 3.35
OSCC	32.75 ± 3.03	6.02 ± 0 .43	7.04 ± 0.67	17.31 ± 1.55
	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
Control	19.98 ± 0.81	2.93 ± 0.79	9.74 ± 0.53	32.18 ± 5.53
OSCC	32.75 ± 3.03	6.02 ± 0 .43	7.04 ± 0.67	17.31 ± 1.55
	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

$P < 0.05$ significant, $P < 0.001$ highly significant

Table 3 Evaluation and comparison of mean ± SD values for salivary and serum MDA and GSH in well differentiated and moderately differentiated OSCC patients

Groups	MDA		GSH	
	Saliva (nmol/dL)	Serum (nmol/mL)	Saliva (nmol/dL)	Serum (nmol/dL)
Well differentiated OSCC	33.04 ± 3.54	6.12 ± 0.36	6.90 ± 0.75	16.84 ± 1.48
Moderately differentiated OSCC	32.47 ± 2.43	5.92 ± 0.49	7.18 ± 0.59	17.78 ± 1.62
	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$

$P > 0.05$ insignificant

Table 4 Evaluation and comparison of mean ± SD values for salivary and serum MDA and GSH by dysplasia grade in oral leukoplakia group

Groups	MDA		GSH	
	Saliva (nmol/dL)	Serum (nmol/mL)	Saliva (nmol/dL)	Serum (nmol/dL)
Mild dysplasia	20.57 ± 1.30	3.29 ± 0.26	9.03 ± 1.31	21.89 ± 4.35
Moderate dysplasia	20.93 ± 1.20	3.49 ± 0.51	8.62 ± 0.82	21.48 ± 2.52
Severe dysplasia	21.29 ± 1.14	3.14 ± 0.49	8.14 ± 1.38	20.78 ± 1.90
	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$

$P > 0.05$ insignificant

statistically significant ($P > 0.05$) (Table 4).

Discussion

ROS are chemical species that either possess free radicals, contain at least one unpaired electron or are composed of reactive non-radical compounds (19,20). High endogenous levels of pro-oxidants and deficiencies in antioxidants levels lead to OS, which causes accumulation of ROS, inducing cellular injury, oxidizing cellular macromolecules such as DNA and playing a crucial role in carcinogenesis (21,22).

Normal cells can be transformed into malignant cells due to oxidative modification. These transformed tumor cells produce high levels of ROS, which in turn increases lipid peroxidation levels. Owing to high cytotoxic properties, lipid peroxidation products such as MDA modulate cell growth by activating signal transduction pathways, therefore acting as tumor promoters and co-carcinogenic agents (8,12).

Due to the very short life of free radicals, their direct measurement is impractical. Indirect methods of OS evaluation include estimation of secondary lipid peroxidation products, such as MDA. Hence, the extent of lipid peroxidation and free radical mediated damage are assayed by serum and salivary MDA levels (23).

GSH is the most abundant, soluble non-enzymatic antioxidant. It has multifunctional roles as a substrate for glutathione S-transferases (GSTs) and glutathione perox-

idases (GPx) during the detoxification of lipid peroxides. It catalyzes decomposition of ROS and prevents its accumulation. It also maintains the redox state of protein sulfhydryls that are necessary for DNA repair (24,25).

The present study was specifically meant to evaluate salivary and serum MDA and GSH levels in controls, and in oral leukoplakia and OSCC patients.

Salivary and serum levels of MDA increased significantly ($P < 0.05$) from healthy controls to oral leukoplakia patients, and a highly significant increase ($P < 0.001$) was seen in oral cancer patients. However, the change was not significant ($P > 0.05$) when well and moderately differentiated OSCC patients were compared.

Lipid peroxidation causes profound changes in the function and structural integrity of the cell membrane. High levels of lipid peroxidation were reported in a study conducted in a baby hamster kidney cell line and its malignant counterpart transformed by polyomavirus, thus suggesting that lipid peroxidation levels are elevated in precancerous lesions and oral cancer (26).

Smoking enhances lipid peroxidation in the saliva. Saliva in tobacco chewers contains tobacco-derived carcinogens such as tobacco-specific nitrosamines and lipid peroxidation products (27,28). Tobacco use results in local exposure of the oral mucosa to nicotine and the heavy metals present in it. Due to the release of nicotine from the mucous membranes and absorption of swallowed nicotine, its continuous absorption takes place even after the tobacco is discarded from the mouth; therefore, it can diffuse from the site of generation and inflict damage at remote locations (29,30). With continuous local irritation by tobacco and the genotoxic effects of nicotine accumulation in saliva, injuries related to chronic inflammation and OS occurs. As a result, oxidative damage is extended to human major and minor salivary glands (28,29). Degenerative changes in more than 40% of minor salivary glands have been observed at the sites of chronic tobacco placement in intense smokeless tobacco users (31). According to a study by Ginzkey et al. (29), DNA migration and damage occurs in parotid gland cells, induced by significant dose-dependent quantities of nicotine. PH-dependent passive diffusion through the oral mucosa exposes small salivary glands found in the submucosa of the lips and palate (29). This sustained inflammatory and oxidative tissue injury leads to larger amounts of oxidant generation, which are able to damage healthy neighboring epithelial and stromal cells over a long period of time, which may contribute to a worsening of the injury, ultimately leading to carcinogenesis (28,32).

In addition, tobacco is consumed in a variety of different ways (smoking, chewing), generating free radicals including ROS that lead to oxidative DNA damage to the surrounding tissues (13,27). Tobacco consumption leads to increase in nicotine exposure, and causes heat generation during smoking and pH changes during chewing, affecting bodily fluids such as blood and saliva and resulting in the formation and stabilization of free radicals (23,29). Thus, tobacco consumption causes overproduction of ROS and free radicals that enhance lipid peroxidation levels, and serum and salivary MDA levels are thereby increased, as seen in oral leukoplakia and OSCC patients.

Increases in MDA levels (in saliva and serum) are not only due to tobacco consumption, but also as a consequence of the magnitude of oxidative stress, supporting the hypothesis that cancer cells have markedly altered ROS metabolism, leading to production of large amounts of ROS as compared to non-neoplastic cells, and the suppression of the antioxidant system that mediate body's defense mechanisms (13,22).

Thus, increased levels of MDA in oral leukoplakia and OSCC patients reflect interactions with various carcinogenic agents, which confirms increased lipid peroxidation and OS in these patients (9,33). However, aggravated free radical damage plays a causative role in carcinogenesis due to reduced activity of antioxidants that fail to cope with elevated OS levels, as observed in our study with significant depletion of GSH levels (34).

On the other hand, a highly significant ($P < 0.001$) decrease was seen in serum and salivary GSH levels from controls to oral leukoplakia to OSCC patients. The use of tobacco may have suppressed the production of antioxidant enzymes, which was evident among oral leukoplakia and OSCC patients, who were tobacco users. Therefore, the OS antioxidant enzyme equation was adversely affected in tobacco consuming patients (35). Furthermore, the decreased GSH levels may be attributed to increased utilization in detoxification of carcinogens, scavenging of free radicals and to counteract prevailing oxidative conditions caused by increase in ROS and pro-oxidants (12). Several studies have reported elevated lipid peroxidation and decreases in antioxidant status in oral cancer (12,22,35,36).

In contrast to the present study, oral pre-cancer and cancer patients have reported increased serum levels of GSH (19,37,38). The increased levels of GSH in these patients reflect the increased detoxification capacity and resistance in response to the cytotoxic substances released by carcinogen-altered cells (19).

Our findings indicated significant lowering of salivary GSH in oral leukoplakia patients and oral cancer

patients, as compared to controls. Singh et al. (39) and Shivshankar et al. (40) reported observations that are in-line with our findings. In contrast, higher salivary levels of GSH were reported by Almadori et al. (25) in patients with oral and pharyngeal cancers. The increase in salivary GSH in these patients is due to a modified salivary environment, as GSH is actively secreted in saliva in response to increased levels of carcinogens but their levels are inconsistent. They concluded that salivary GSH concentration ranges too widely to be used as a diagnostic marker in oral or pharyngeal SCC (25).

Patel et al. (23) evaluated plasma levels of lipid peroxidation, thiol and total antioxidant status in healthy controls and oral cancer patients. Elevated risk of oral cancer development was seen in those controls who had higher tobacco exposure and lower thiol levels. Lipid peroxidation levels above the cut-off level along with total antioxidant status and thiol levels below the cutoff levels, predict poor overall survival in patients. Thiols such as GSH interfere with the complex carcinogenic process by conjugating with electrophilic carcinogens (all carcinogens are highly electrophilic, and may attack electron rich sites on macromolecules, particularly DNA in target cells), thereby preventing tumor initiation and defending against oxidative stress (41).

Lower levels of thiol indicate more of an oxidized state in tobacco users. Tobacco consumption causes depletion of thiol (-SH groups) in the redox equation leading to a decrease in buffer activity of antioxidants, and thus tobacco use leads to cancer progression (23). Thus, for predicting the risk of oral carcinogenesis and overall survival of oral cancer patients, lipid peroxidation and thiol levels are useful biomarkers.

Saliva plays a pivotal role in OSCC pathogenesis (42). Saliva has powerful anticarcinogenic effects due to the presence of various enzymes and the salivary antioxidant system. The oral cavity is equipped with enzymatic and nonenzymatic salivary antioxidant systems (42). The enzymatic component includes superoxide dismutase (SOD), peroxidase, catalase (CAT), GST and GPx, while the nonenzymatic component includes uric acid and GSH (43). As discussed above, tobacco consumption plays a significant role in oral carcinogenesis by generating various free radicals and ROS, such as hydroxyl radicals (OH), superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) (13,23). Saliva constitutes a first line of defense against free radical-mediated OS by inhibiting the production of ROS, superoxide free radicals (O_2^-) and H_2O_2 (44). Enzymes such as SOD are the most effective, intracellular, tumor-suppressing antioxidant enzyme that catalyzes the dismutation of oxygenated free radicals to less-reactive species, and therefore regulates cellular redox state and leads to tumor growth retardation (45). Peroxidase, CAT or GPx prevents carcinogenesis by converting H_2O_2 produced by SOD into non-harmful molecular oxygen and water (34). GST, another pivotal anticancer salivary enzyme, protects initiation of carcinogenesis by detoxifying electrophilic carcinogenic intermediates and eliminating chemical carcinogens (19,42).

The other important salivary component, GSH has a redox buffering capacity due to its ability to regenerate the most important antioxidants back to their active forms (10). Nishioka et al. conducted the Ames test and found that saliva inhibited the genotoxic effects of benzopyrene and condensate of cigarette smoke that are well-known oral cancer inducers. Further credence of saliva as an anticarcinogenic medium was demonstrated by Dayan et al., who conducted an animal study where he found that desalivated group of rats were more affected with pathological changes in shorter duration, as compared to controls on administration of 4-Nitroquinoline 1-oxide (4NQO), which is a local carcinogen. Thus, it can be concluded that saliva, detoxifies 4NQO and benzopyrene and protects against carcinogenesis (46).

The diagnostic efficacy of saliva can be attributed to the fact that there is an ever continuous and intimate proximity of saliva to oral neoplasms and premalignant lesions. Salivary oxidative alterations can be non-invasively monitored for antioxidants, oxidized DNA and proteins and can be used to identify high risk cases of oral cancer (42). In recent years, increasing amounts of research have been performed on saliva owing to the ease and safety with which it can be collected as compared to serum (28). Future research should aim to establish saliva as a diagnostic fluid for primary screening and monitoring of disease progression in oral cancer cases due to its easy accessibility. Further benefits are that the salivary collection procedure is non-invasive, can be undertaken at low cost and without involvement of skilled personnel (47).

ROS and free radicals have predominant deleterious role in inducing and promoting carcinogenesis. The present study suggests a role for MDA and GSH as diagnostic biomarkers and innovative tools to monitor OS and their impact on prognosis of oral premalignancy and malignancy. Saliva can be used to determine the impact of redox imbalance on the progression of OSCC, but for this purpose, stronger correlation studies with a larger sample size are necessary before establishing saliva as a reliable laboratory tool.

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