Original Article

Salivary malondialdehyde in oral submucous fibrosis – A marker for oxidative damage

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Abstract

Background: Oral submucous fibrosis (OSMF) is a potentially malignant disorder of oral mucosa and its malignant transformation rate accounts to about 7%–13%. Oxidative damage and lipid peroxidation plays an important role in OSMF. Lipid peroxidation has not been widely investigated in OSMF patients with respect to clinical staging and histopathological grading. As human saliva is a diagnostic fluid which can be obtained in a noninvasive procedure as compared to the blood for serum analysis, the present study was aimed at evaluating the salivary malondialdehyde (MDA) levels in OSMF and comparison with respect to clinical staging and histopathological grading.

Aim: This study aims to evaluate salivary MDA levels in OSMF and compare the levels with respect to clinical and histopathological grading systems.

Materials and Methods: Forty cases of clinically diagnosed and histopathologically proven cases of OSMF were included for the purpose of this study. As controls 40 age-matched individuals without any systemic disease were selected. Unstimulated whole saliva was collected from each individual, centrifuged and frozen at − 20°C until analysis. Lipid peroxidation products MDA were analyzed by thiobarbituric acid reaction.

Results: Salivary MDA levels were significantly increased in OSMF patients compared to controls. The progressively increased salivary MDA levels showed a positive correlation with the clinical stages and histopathological grades of OSMF and the results were statistically significant.

Conclusion: The increased salivary MDA levels in OSMF patients compared to the control group suggests an increased oxidative stress levels in the potentially malignant disorders such as OSMF. The mean salivary MDA levels were increased significantly as the clinical stage and histopathological grade of OSMF advances, suggesting MDA to be used as a reliable biochemical marker and also a prognostic marker to assess the extent of oxidative damage in OSMF.

Keywords: Malondialdehyde, oral submucous fibrosis, prognostic biochemical marker, saliva, thiobarbituric acid

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INTRODUCTION

Oral submucous fibrosis (OSMF) is a chronic, potentially malignant disorder of oral mucosa which was first described by Schwartz in 1952.[1] In 1966, it was defined by Pindborg as "An insidious, chronic disease affecting any part of the oral cavity and sometimes the pharynx. Although occasionally preceded by and/or associated with vesicle formation, it is always associated with juxta-epithelial inflammatory reaction followed by a fibroelastic change of the lamina propria, with epithelial atrophy leading to stiffness of the oral mucosa and causing trismus and inability to eat."[2]

OSMF is predominantly seen in Southeast Asian countries along India. The rampancy and prevalence rate of OSMF particularly in the Indian subcontinent is about 0.2%–0.5%.[3] The major effect of this disease lies in its inability to open the mouth and possessing the highest malignant transformation rate which accounts to about 7%–13%.[4]

The etiopathogenesis of OSMF is much more complex and involves multiple factors such as consumption of chillies, nutritional deficiencies, betel quid chewing, genetic susceptibility, altered salivary constituents, autoimmunity and collagen disorders.[5] Among all, betel quid chewing with or without tobacco constitutes a major risk factor.[6]

A betel quid constituent such as areca nut and catechu along with tobacco generates reactive oxygen species (ROS) in substantial amounts in the oral cavity which are implicated in multistage carcinogenesis. These ROS and free radicals (such as H_2O_2 ions, hydroxyl radical and superoxide anions which are highly reactive due to the presence of unpaired electrons in the valence shell) can inflict direct oxidative damage to the lipids present in the oral mucosal cell wall, through lipid peroxidation.[7,8]

MDA is one of the final byproducts of lipid peroxidation.[8] The levels of MDA can be evaluated in various samples such as serum, plasma, tissues and saliva. Saliva is a diagnostic fluid which can be obtained in a noninvasive procedure as compared to blood for serum analysis. In addition, blood concentrations of many components are reflected in saliva.[9] Numerous studies have suggested that salivary proteins may be potentially valuable for diagnosis and/or prognosis of human disease. Hence, markers for oxidative stress can be evaluated in saliva.[10]

At present, MDA is emerging as a valuable biomarker in the diagnosis and treatment plan of many conditions such as ovarian cancer, breast cancer, cardiovascular diseases, oxidative stress in pregnancy and hypertension[11] However,

only few studies have been reported about MDA levels in head and neck regions.

Earlier studies showed increased MDA levels in oral squamous cell carcinoma (OSCC) and potentially malignant disorders such as leukoplakia, OSMF. Hence, the present study was carried out to compare the salivary MDA levels pertaining to clinical and histopathological stages of OSMF. This is probably the first study which was conducted in saliva for the estimation of MDA levels in OSMF and its comparison with both clinical and histopathological stages.

MATERIALS AND METHODS

Study design and case selection
A total of 80 individuals of which 40 clinically diagnosed and histopathologically proven cases of OSMF who reported to the outpatient department of our institution and 40 age-matched healthy individuals without any habits and underlying systemic diseases were included for the purpose of this study. The study was approved by the ethical committee of our institute.

Collection of sample
After obtaining an informed consent, the study group and the healthy individuals were asked not to eat or drink or chew gum before collection of saliva. The individuals were then asked to allow saliva to pool in the floor of the mouth and spit into a plastic container. The sample was centrifuged at 2000 rpm for 10 min and the supernatant was collected in a sterile vial, labeled and stored at − 20°C until analysis.

Categorization of individuals with OSMF
The OSMF group was categorized clinically before obtaining the saliva sample into three groups according to Kiran Kumar *et al*.[5] After obtaining the sample, a punch biopsy was taken and the individuals were categorized histopathologically according to Utsunomiya *et al*.[3]

Of 40 OSMF patients, according to clinical staging, Stage I were 10 cases (25%), Stage II-16 cases (40%) and Stage III – 14 cases (35%) [Table 1]. Histopathologically, the cases were graded as early Stage – 11 cases (27.5%), intermediate stage– 17 cases (42.5%) and advanced Stage– 12 cases (30%) [Table 2].

Table 1: Salivary malondialdehyde levels in clinical staging

Clinical stages MDA (nM/ml)	*n*	Mean	SD	SEM
Stage 1	10	0.2860	0.03098	0.00980
Stage 2	16	0.3569	0.04254	0.01063
Stage 3	14	0.4157	0.03756	0.01004

SD: Standard deviation, SEM: Standard error of mean, MDA: Malondialdehyde

Thiobarbituric acid-trichloroacetic acid method

The estimation of MDA was done by the thiobarbituric acid-trichloroacetic acid (TBA-TCA) method, wherein the salivary samples were heated with TBA-TCA reagent in a water bath for 20 min. The reagent consisted of 20% TCA, 0.5% TBA and 2.5N HCL. After cooling, the solution was centrifuged at 2000 rpm for 10 min and the precipitate was removed. The absorbance of the supernatant was determined at 532 nm against blank that contained all the reagents minus the biological sample. The MDA equivalents of the sample were calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Statistics

Statistical analysis of the data was performed by using the IBM® SPSS® Statistics 20 (where IBM is a company). Student's *t*-test was done to compare OSMF and control group. Bonferroni test was used in conjunction with an ANOVA for comparison between multiple unequal groups.

RESULTS

The mean salivary MDA levels of OSMF and control groups were compared, which revealed a highly significant rise in the MDA levels of OSMF group compared to the control group with the $P < 0.05$ [Figure 1]. The salivary MDA levels of all the clinical stages and histopathological grades of OSMF group were illustrated in the Tables 1 and 2, respectively.

On comparison of the three clinical stages by One Way ANOVA test, there was a significant difference in between and also within the stages. When multiple comparisons were done using a *post hoc* Bonferroni test, statistically significant difference was observed among all three clinical stages and the levels increased as the stage advanced [Tables 3 and 4 and Figure 2].

Table 2: Salivary malondialdehyde levels in histopathological grades

| Histopathological grades MDA (nM/ml)	*n*	Mean	SD	SEM
Grade 1	11	0.3009	0.03208	0.00967
Grade 2	17	0.3459	0.04001	0.00970
Grade 3	12	0.4333	0.03055	0.00882

SD: Standard deviation, SE: Standard error, MDA: Malondialdehyde

**Table 3: ANOVA test showing comparison of salivary malondialdehyde levels in clinical staging**

|	MDA				
	Sum of squares	df	Mean square	F	Significant
Between groups	0.098	2	0.049	33.622	0.000
Within groups	0.054	37	0.001		
Total	0.152	39			

MDA: Malondialdehyde

In the same way, comparison of three histopathological grades using one-way ANOVA test showed a significant difference between and within the grades and on performing multiple comparisons between the three grades using Bonferroni *post hoc* test the results were found to be statistically significant and salivary MDA levels increased as the histopathological grade advanced [Tables 5 and 6 and Figure 3].
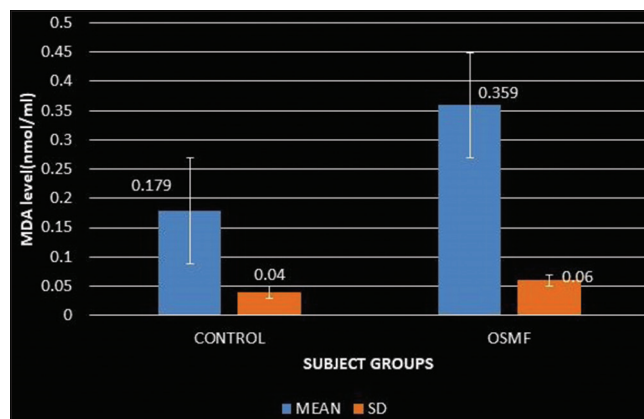
Figure 1: Graph illustrating comparison of salivary malondialdehyde levels between oral submucous fibrosis and control groups
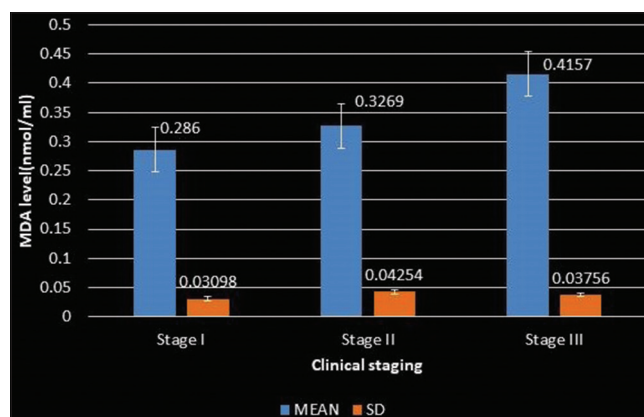
Figure 2: Graph illustrating comparison of salivary malondialdehyde levels between Clinical stages of oral submucous fibrosis
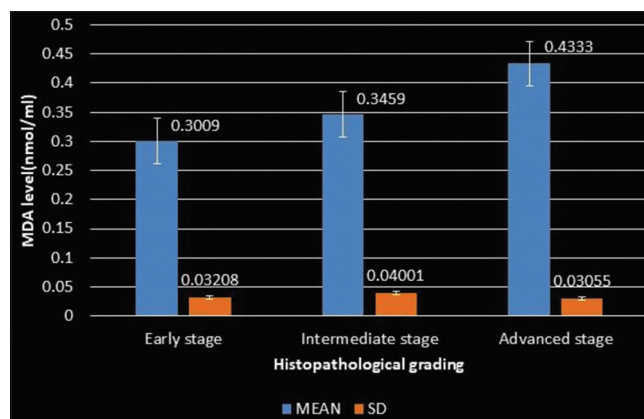
Figure 3: Graph illustrating comparison of salivary malondialdehyde levels between Histopathological grades of oral submucous fibrosis

Table 4: Comparison of salivary malondialdehyde levels between patients with different clinical stages of oral submucous fibrosis

Stage	Stages	Mean difference (I-J)	SE	Significant	95% CI	
					Lower bound	Upper bound
Stage 1	Stage 2	−0.07087*	0.01542	0.000	−0.1095	−0.0322
	Stage 3	−0.12971*	0.01584	0.000	−0.1694	−0.0900
Stage 2	Stage 1	0.07087*	0.01542	0.000	0.0322	0.1095
	Stage 3	−0.05884*	0.01400	0.000	−0.0939	−0.0237
Stage 3	Stage 1	0.12971*	0.01584	0.000	0.0900	0.1694
	Stage 2	0.05884*	0.01400	0.000	0.0237	0.0939

*The mean difference is significant at the 0.05 level. Dependent variable: MDA. *Post hoc* test: Bonferroni. SE: Standard error, CI: Confidence interval, MDA: Malondialdehyde

Table 5: ANOVA test showing comparison of salivary malondialdehyde levels in histopathological grades of oral submucous fibrosis

	MDA				
	Sum of squares	df	Mean square	F	Significant
Between groups	0.106	2	0.053	42.606	0.000
Within groups	0.046	37	0.001		
Total	0.152	39			

MDA: Malondialdehyde

DISCUSSION

OSMF is a chronic disease affecting the oral cavity. The main etiological agent which is responsible for this condition is chewing betel nut which affects the oral cavity through ROS.[7] ROS are the chemical species constituting both free radicals (possessing at least one unpaired electron) and reactive non radical compounds. These include superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (HO.), peroxyl radical (ROO.), nitric oxide (NO), peroxynitrite (ONOO-) and singlet oxygen (O2). Excess levels of these radicals produce similar biological effects and so they are grouped under the term ROS.[8] In OSMF, there are three main targets for these ROS to act upon which includes bilipid layer of cell membrane, DNA and proteins.[12]

Chewing betel quid was known to be the prime etiological agent in the causation of OSMF through varied micromolecular mechanisms such as altered collagen metabolism, altered levels of micronutrients, inflammation and lipid peroxidation.[13] Lipid peroxidation is a chain reaction initiated by the hydrogen abstraction or addition of an oxygen radical, resulting in the oxidative damage of polyunsaturated fatty acids (PUFA). Since PUFA are more sensitive than saturated ones, the activated methylene (RH) bridge represents a critical target site. The presence of a double-bond adjacent to a methylene group makes the methylene C-H bond weaker and therefore the hydrogen in more susceptible condition to abstraction. This leaves an unpaired electron on the carbon, forming a carbon-centered radical, which is stabilized by a molecular rearrangement of the double bonds to form a conjugated diene which
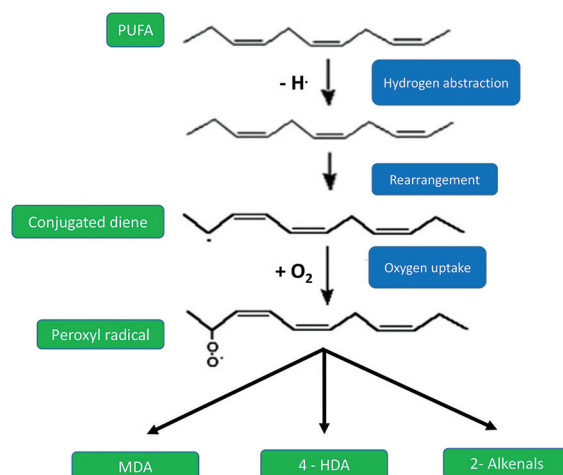
Figure 4: Flow chart depicting the process of lipid peroxidation

then combines with oxygen to form a peroxyl radical. The peroxyl radical itself is capable of abstracting a hydrogen atom from another PUFA thereby starting a chain reaction which finally leads to the formation of aldehydes such as MDA, hydroxynonenal, and other dialdehydes as secondary and end products of lipid peroxidation.[14] [Figure 4].

In the pathogenesis of OSMF, cellular metabolism of Betel nut or Betel quid components generate ROS, such as superoxide anion radicals (O2-) and hydrogen peroxide (H_2O_2) at pH >9.5. These ROS attack the cell membrane which finally leads to formation of aldehydes i.e., MDA.[7,12] MDA is a toxic compound that reacts with DNA to form covalently-bonded adducts with deoxyadenosine and deoxyguanosine, an event that can cause a mutagenic transformation within the DNA by altering their chemical behavior and possibly contributing to carcinogenesis and mutagenesis.[12] [Figure 5].

Saliva has been found to contain constituents that reflect the diseased or physiological state of the human body, and hence could be utilized for diagnostic purposes. The search for reliable salivary biomarkers for early detection of OSCC has developed rapidly, based on the fact that collecting saliva is relatively easy and noninvasive, compared to the collection of blood.[9] In the present study, salivary MDA

Table 6: Comparison of salivary malondialdehyde levels between different histopathological grades of oral submucous fibrosis

Grade	Grades	Mean difference (-J)	SE	Significant	95% CI	
					Lower bound	Upper bound
Grade 1	Grade 2	−0.04497*	0.01367	0.007	−0.0793	−0.0107
	Grade 3	−0.13242*	0.01475	0.000	−0.1694	−0.0954
Grade 2	Grade 1	0.04497*	0.01367	0.007	0.0107	0.0793
	Grade 3	−0.08745*	0.01332	0.000	−0.1209	−0.0541
Grade 3	Grade 1	0.13242*	0.01475	0.000	0.0954	0.1694
	Grade 2	0.08745*	0.01332	0.000	0.0541	0.1209

*The mean difference is significant at the 0.05 level. Multiple comparisons. Dependent variable: MDA. *Post hoc* test: Bonferroni. SE: Standard error, CI: Confidence interval
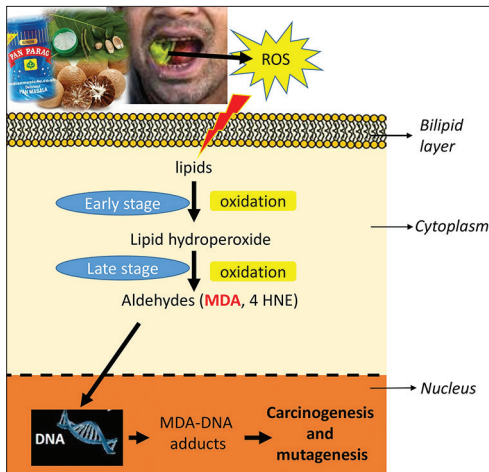
Figure 5: Flow chart depicting the role of constituents of betel quid in carcinogenesis through malondialdehyde

levels of OSMF group were compared with the control group and it was shown that the levels are significantly higher in OSMF group which is in accordance with the previous studies conducted on both serum and salivary MDA levels in OSMF.[12,15-22] Shetty *et al*.[16] reported an increase in salivary MDA levels in OSMF as grading progressed. Similar observations were reported in our study where the clinical staging and histopathological grading is according to Kiran Kumar *et al*.[5] and Utsunomiya Tilakartne *et al*.[3] respectively. According to Chole *et al*.,[18] serum MDA levels were significantly increased in oral cancer and precancer group compared to healthy individuals and the results of precancer group coincided with the present study. Tejaswi *et al*.[12] studied serum MDA levels with clinical stages and found there is no significant increase in serum MDA levels. In contrast, the present study was done on salivary MDA levels and found a significant increase in the salivary MDA levels as the clinical stage progressed.

Metkari *et al*.[22] found an increased serum MDA levels in OSMF patients with advancement in clinical staging. However, on comparison with histopathological grading the levels were not increased as the grade advanced. In contrast present study revealed an increased salivary MDA levels with both clinical staging and histopathological grading. The mean MDA levels varies in most of the studies

which may be due to multiple reasons such as age, mode of habit or different styles of consumption of tobacco and betel nut, alcohol consumption and methodological differences.

CONCLUSION

From the present study, it was determined that by salivary estimation of MDA, one can assess the degree of oxidative damage occurred in the tissue in OSMF. The increase in MDA levels as the clinical and histopathological stages progressed shows that MDA can be used as a prognostic marker in the treatment plan of OSMF. Furthermore, long-term studies with larger sample size with proper follow-up are required to determine the actual role of MDA in carcinogenesis.

Conflicts of interest
There are no conflicts of interest.

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