

Short Communications

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Salivary Malondialdehyde as a Marker of Oxidative Stress in Tobacco Chewers, with and without Oral Submucous Fibrosis: A Comparative, Cross-Sectional Biochemical Study

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Abstract

Objective: This study aimed to evaluate and compare salivary Malondialdehyde (MDA) levels in tobacco chewers with and without oral submucous fibrosis, and healthy individuals, and correlate the levels with stages of oral submucous fibrosis. **Methods:** This cross-sectional study was conducted in ITS-Centre for Dental Studies and Research, Ghaziabad, India which included 60 subjects divided into three groups: Group A: Healthy controls (n=20) Group B: Tobacco chewers with clinically and histopathologically confirmed Oral Submucous Fibrosis (OSMF) (n=20) Group C: Tobacco chewers without OSMF (n=20). Unstimulated whole saliva was collected, centrifuged, and stored at -20°C . Salivary MDA levels were estimated by enzyme-linked immunosorbent assay (ELISA) using thiobarbituric acid reactive substances (TBARS). **Results:** Salivary MDA levels were significantly higher in oral submucous fibrosis patients (mean \pm SD: 49.57 ± 25.91 mmol/L) compared to tobacco chewers without oral submucous fibrosis (21.21 ± 7.76 mmol/L) and healthy individuals (10.96 ± 2.90 mmol/L), with $p < 0.001$. A statistically significant positive correlation was found between MDA levels and stages of oral submucous fibrosis, indicating increased oxidative stress with disease progression. **Conclusion:** Elevated salivary MDA levels in oral submucous fibrosis patients reflect increased oxidative stress and its correlation with disease severity.

Keywords: Malondialdehyde- Oral Submucous Fibrosis- Oxidative Stress- Saliva- Prognostic Marker- ELISA

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Introduction

Oral Submucous Fibrosis (OSMF) is a chronic, progressive, and potentially malignant disorder of the oral cavity, prevalent in South and Southeast Asia [1]. It is characterized by juxta-epithelial fibrosis, leading to stiffness of oral mucosa, trismus, and an increased risk of malignant transformation, with reported rates between 7% and 13% [2].

India reports high rates of tobacco usage, with approximately 25.9% of adult population reported for smokeless tobacco consumption [3]. This contributes significantly to the burden of oral cancers, which account for nearly half of all malignancies in Indian males and one-fourth in females [4]. The absorption of nicotine through oral mucosa is nearly double in smokeless tobacco users compared to smokers [5]. Nicotine's interaction with acetylcholine receptors results in neurochemical changes that promote addiction, while other harmful components in tobacco induce oxidative stress, tissue damage, and apoptotic cell death [6].

The etiopathogenesis of OSMF is multifactorial, with areca nut being the principal carcinogen. Other contributing factors include betel quid chewing, nutritional deficiencies, and genetic susceptibility [7]. Alkaloids in areca nut, such as arecoline induce collagen synthesis and inhibit its degradation, contributing to progressive fibrosis [8]. Increased lysyl oxidase activity and reactive oxygen species (ROS) production further exacerbate tissue damage through lipid peroxidation and DNA alterations [1].

OSMF frequently affects buccal mucosa, soft palate, uvula, and floor of the mouth. Early symptoms include burning sensation, blistering, ulceration, and restricted mouth opening. With progression, the mucosa appears blanched and marble-like with fibrous bands [1]. Despite lack of overt epithelial dysplasia, OSMF exhibits high malignant potential due to epithelial atrophy and submucosal fibrosis that compromise vascularity and mucosal integrity [9].

Although biopsy remains the gold standard for histopathological diagnosis, its invasive nature and poor

patient compliance limit widespread acceptance. Saliva is a biological fluid which offers a promising alternative for the evaluation of disease biomarkers. It reflects systemic changes and has been referred to as the “mirror of the body” due to its diagnostic potential [10]. Salivary diagnostics have shown utility in oral diseases, including potentially malignant disorders and cancers [11].

Among the various biomarkers of oxidative stress, Salivary Malondialdehyde (MDA) is widely recognized. It is a reactive aldehyde formed during the breakdown of polyunsaturated lipids by ROS, leading to DNA adduct formation and mutagenic changes [3]. However, there is limited literature exploring the correlation between salivary MDA levels and clinical and histopathological staging of OSMF. And also limited literature which evaluates MDA levels in tobacco chewers prior progression to OSMF.

Therefore, the present study aimed to evaluate and compare salivary MDA levels in tobacco chewers with and without OSMF, and in healthy individuals, and to determine the association of MDA with the severity of OSMF. This investigation seeks to establish MDA as a reliable, non-invasive prognostic biomarker for disease monitoring and early detection.

Materials and Methods

Study Design

This cross-sectional study was conducted in the Department of Oral Medicine and Radiology, ITS Dental College, Murad Nagar, Ghaziabad, India. Ethical clearance was obtained from the Institutional Ethical Committee (Approval No: ITSCDSR/IEEC/2022-2025/OMR/02). All procedures were conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each participant prior to data collection.

Study Population and Groups

A total of 60 participants aged 18 to 60 years were categorized into three groups (n = 20 each) based on clinical history and oral examination:

Group A: Healthy individuals without any tobacco-related habits (control group)

Group B: Tobacco chewers with clinically and histopathologically confirmed Oral Submucous Fibrosis (OSMF)

Group C: Tobacco chewers without any clinical signs of OSMF

Detailed history regarding oral and general health, habit frequency and duration, and any medication or systemic conditions was obtained using a structured questionnaire.

Inclusion Criteria

- i. Adults aged 18–60 years
- ii. Healthy controls without any deleterious habits (Group A)
- iii. Clinically and histologically diagnosed cases of OSMF (Group B)
- iv. Habitual tobacco chewers without OSMF (Group C)

Exclusion Criteria

- i. History of smoking or any systemic inflammatory/autoimmune diseases
- ii. Presence of oral lesions other than OSMF
- iii. Patients undergoing treatment for OSMF
- iv. Immunocompromised individuals or pregnant/lactating women

Saliva Collection Protocol

Unstimulated whole saliva samples were collected in the morning hours (9–11 AM) to minimize diurnal variations. Participants were asked to refrain from eating, drinking, chewing gum for at least 30 minutes prior to collection. Saliva was allowed to pool naturally in the mouth and drooled into sterile, pre-labelled Eppendorf tubes. Samples were immediately centrifuged at 4,000 rpm for 15 minutes at 4°C to remove debris. The clear supernatant was aliquoted and stored at –20°C until analysis.

Assessment of Salivary Malondialdehyde

Salivary MDA levels were quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Bioassay Technology Laboratory, China) with a detection sensitivity of 0.14 nmol/mL. Assay was performed according to the manufacturer’s (Figure 1) instructions. Initially, 40 µL of saliva sample was mixed with anti-MDA antibody and streptavidin–HRP, incubated at 37°C for 60 minutes (Figure 2), and washed. Following incubation, the reaction was stopped and absorbance was read at 450 nm using a microplate reader. A standard curve was used to determine MDA concentrations (Figure 3).

Results

The present study evaluated salivary malondialdehyde (MDA) levels in 60 participants divided into three equal groups: Group A (healthy individuals with no tobacco habit), Group B (tobacco chewers with clinically and histopathologically confirmed oral submucous fibrosis [OSMF]), and Group C (tobacco chewers without OSMF). The mean age of participants was 44.20 ± 11.75 years in Group A, 45.65 ± 7.73 years in Group B, and 42.95 ± 8.23 years in Group C. Statistical analysis using the Kruskal–Wallis H test showed no significant difference in age distribution among the groups (p = 0.612). Gender distribution revealed a higher proportion of males in both tobacco-chewing groups, but the difference was not statistically significant (p = 0.619).

Salivary MDA levels were assessed using an ELISA-based method. A clear progressive increase in salivary MDA concentration was observed across the groups. Group A (healthy controls) exhibited the lowest mean MDA level of 10.96 ± 2.90 nmol/L. Group C (tobacco chewers without OSMF) showed moderately elevated MDA levels with a mean of 21.21 ± 7.76 nmol/L, while Group B (tobacco chewers with OSMF) presented with the highest mean MDA concentration of 49.57 ± 25.91 nmol/L (Table 1). The Kruskal–Wallis test confirmed that this intergroup difference in MDA levels was statistically significant (p < 0.001), indicating a strong association

Table 1. Descriptive Statistics of Salivary Malondialdehyde (MDA) Values Evaluated by ELISA in Three Study Groups

	Mean	SD	SE	Minimum	Maximum
Group A (Healthy)	10.96	2.9	0.64	6.1	18.2
Group B (Tobacco chewing + OSMF)	49.57	25.91	5.79	21.04	109.38
Group C (Tobacco chewing without OSMF)	21.21	7.76	1.73	12.54	45.1

Table 2. Intragroup Comparison of Salivary Malondialdehyde (MDA) Values Evaluated by ELISA Different Grades of OSMF Respectively

Group B (Tobacco chewing + OSMF)	Mean	SD	Kruskal Wallis H test	P value, Significance
Grade 1	26.52	3.41	H =16.913	P < 0.001**
Grade 2	44.06	6.73		
Grade 3	82.88	19.15		

between elevated oxidative stress and the presence of OSMF.

Pairwise comparisons using the Mann–Whitney U test further clarified these differences. A statistically significant difference was noted between Group A and Group B ($p < 0.001$), and between Group B and Group C ($p < 0.001$). Although the mean MDA level in Group C was higher than in Group A, this difference did not reach statistical significance ($p = 0.106$). These findings suggest that while tobacco use alone may slightly elevate MDA levels, the presence of OSMF is associated with a substantial increase in oxidative stress, as reflected by the higher salivary MDA levels.

To assess the correlation between salivary MDA and disease severity, MDA concentrations were also compared within Group B based on staging of OSMF. Participants with Grade I OSMF exhibited a mean MDA level of 26.52 ± 3.41 nmol/L, those with Grade II showed 44.06 ± 6.73 nmol/L, and those with Grade III had markedly elevated levels at 82.88 ± 19.15 nmol/L. The Kruskal–Wallis H test revealed this intragroup difference to be statistically significant ($p < 0.001$), indicating a strong correlation

between increasing clinical severity and rising oxidative stress levels (Table 2).

Subgroup analysis using the Mann–Whitney U test revealed that the differences in MDA levels between Grade I and Grade III ($p < 0.001$), and between Grade II and Grade III ($p < 0.001$) were statistically significant. The difference between Grade I and Grade II was also significant ($p = 0.026$), though to a lesser degree. These results support the observation that salivary MDA levels progressively increase with the advancement of OSMF stages, highlighting its potential as a prognostic biomarker for disease progression.

Discussion

Tobacco use has been deeply entrenched in human history for over 3,000 years, with *Nicotiana tabacum* and *Nicotiana rustica* being its principal sources. Among the numerous toxic compounds it contains, nicotine is the most potent and addictive alkaloid, known to exert systemic effects by acting on nicotinic cholinergic receptors [5]. The commercialization of tobacco-containing pan masala



Figure 1. Human Malondialdehyde Elisa Kit

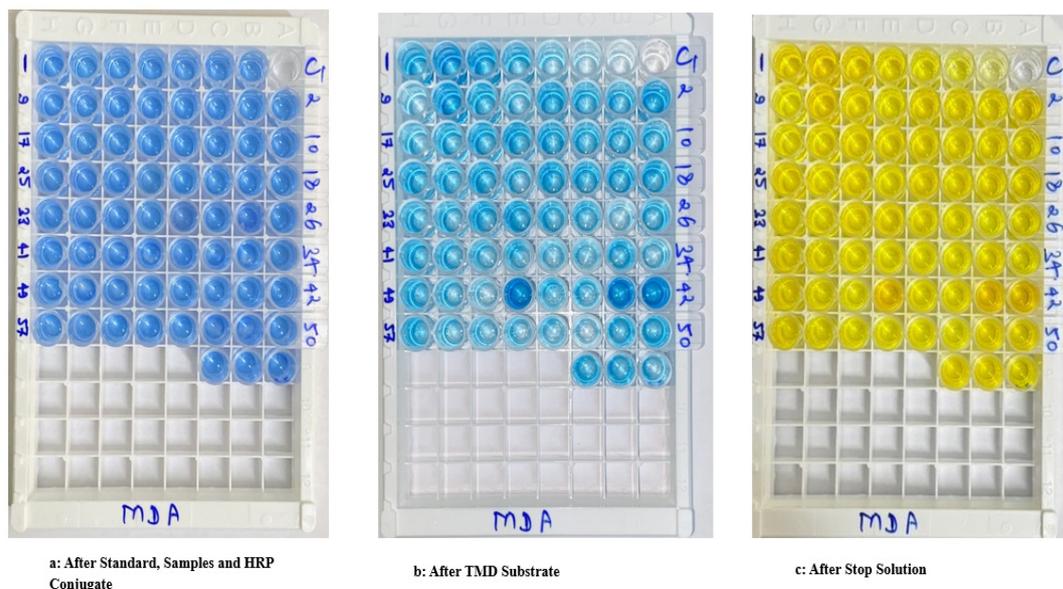


Figure 2. (a) Initially, 40 μ L of saliva sample in each well was mixed with anti-MDA antibody and streptavidin–HRP, incubated at 37°C for 60 minutes, and washed. (b) TMB substrate added in each well (c) 50 μ l Stop Solution was added to each well, the blue color changed into yellow immediately.

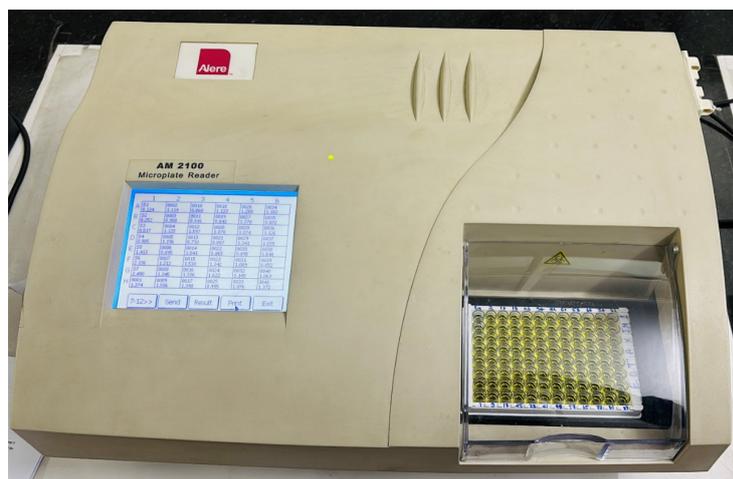


Figure 3. Microplate Reader

in the Indian subcontinent during the 1970s significantly increased the prevalence of oral tobacco use [12].

A major consequence of chronic exposure to tobacco is the development of potentially malignant disorders (PMDs), such as oral submucous fibrosis (OSMF), leukoplakia, and erythroplakia [13]. OSMF, in particular, is a progressive, debilitating condition characterized by fibrosis of the oral mucosa, trismus, burning sensation, and epithelial atrophy. The pathogenesis of OSMF is multifactorial, with areca nut alkaloids and tobacco compounds inducing persistent inflammation, reactive oxygen species (ROS) generation, and eventual fibrotic transformation [7].

Among various biomarkers of oxidative stress, malondialdehyde (MDA) has emerged as one of the most reliable end-products of lipid peroxidation. ROS such as hydroxyl radicals, superoxide, and hydrogen peroxide can oxidize polyunsaturated fatty acids (PUFAs) in cell membranes, resulting in MDA formation [10]. MDA, in

turn, forms adduct with DNA and proteins, contributing to mutagenesis, cellular dysfunction, and oncogenesis [7]. Several studies have implicated elevated MDA levels in a range of systemic and oral disorders including cardiovascular disease, breast and ovarian cancer, and PMDs like Leukoplakia and OSMF [2].

Saliva has gained prominence in recent years as a diagnostic medium for monitoring oxidative stress due to its non-invasive, simple, and cost-effective collection method [10]. Most importantly, saliva contains nearly all systemic biomarkers, including MDA, which makes it a promising alternative for point-of-care diagnostics in oral precancer detection [14].

While previous studies have evaluated salivary or serum MDA levels in OSMF patients, very few have explored the gradation of MDA in patients without visible clinical signs particularly in tobacco chewers without OSMF. The current study is distinctive as it seeks not only to correlate MDA levels with OSMF severity but also to

identify subclinical oxidative stress in habitual tobacco users with no visible signs of a lesion. This offers a novel perspective in OPMD research, positioning salivary MDA as a potential early-warning biomarker for future malignant transformation.

In the current study, the mean MDA levels were found to be highest in tobacco chewers with OSMF (49.57 ± 25.91 mmol/L), followed by tobacco chewers without OSMF (21.21 ± 7.76 mmol/L), and least in healthy individuals (10.96 ± 2.90 mmol/L). The statistically significant difference ($p < 0.001$) between these groups aligns with earlier studies by Shetty et al. [7] and Balasubramaniam et al. [3], who reported similar trends of increasing MDA levels with OSMF severity.

Moreover, the present study demonstrated a clear increase in MDA levels with progressive clinical grading of OSMF: from 26.52 ± 3.41 mmol/L in Grade I to 82.88 ± 19.15 mmol/L in Grade III. This is consistent with the findings of Ganta et al. [2], who reported that MDA levels rise proportionately with the stage of fibrosis.

An especially noteworthy outcome of this study is the elevated MDA levels detected in tobacco chewers without OSMF compared to healthy subjects. This intermediate elevation (21.21 ± 7.76 mmol/L vs. 10.96 ± 2.90 mmol/L) supports the hypothesis that oxidative stress precedes clinical manifestations, and salivary MDA can serve as a preclinical marker. This underlines the novelty of our study, which provides biochemical evidence of subclinical cellular damage in otherwise normal-appearing mucosa.

The pairwise comparison between tobacco chewers with OSMF and those without revealed a statistically significant difference in MDA levels (mean difference = 28.36, $p < 0.001$). This quantifies the oxidative burden during the transition from habit to pathology.

This study emphasizes the need for salivary screening among habitual tobacco chewers even those lacking clinical symptoms to detect early molecular alterations. It opens a window of opportunity for preventive counselling and possible pharmacological intervention using antioxidants. Public health programs can integrate such non-invasive tests for mass screening in high-risk populations.

Furthermore, our findings propose a potential role for salivary MDA in risk stratification. Tobacco chewers with elevated MDA levels but no visible lesions may represent a “subclinical at-risk” population. It uniquely emphasizes the predictive value of salivary MDA in patients who do not yet present with any lesion—an aspect rarely addressed in existing literature.

In conclusion, the current study provides robust evidence that salivary MDA levels are significantly elevated in tobacco chewers, particularly those with OSMF. Importantly, even in the absence of clinical signs, tobacco chewers exhibited significantly higher MDA levels than healthy individuals, suggesting early subclinical oxidative damage. This underscores the role of MDA as a sensitive biomarker for oxidative stress and a potential predictor of disease progression.

Author Contribution Statement

Sylvia Waikhom: Performing of study, sample collection, and manuscript drafting; Akshay Rathore: Designing of study, concept and supervision; Anuradha Yadav: Manuscript review; Parul Tikoo: Data analysis; Qazi Saba: Literature review; Achom Uma: Literature Review.

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Ethical Clearance

Approved by the institute ethical committee. ITSCDSR (Approval No: ITSCDSR/IEC/2022-2025/OMR/02)

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