ORIGINAL ARTICLE

Correlation of Salivary Lactate Dehydrogenase Levels with Histopathological Grades of Oral Cancer

Ruba Odeh 1,2, Ahmed Azhari Salih Mohamedeissa 1,2, Jad Moriss Kazma 1,2, Maher Al Shayeb 1,2*

ABSTRACT

Background

Oral squamous cell carcinoma (OSCC) remains a significant global health challenge with high morbidity and mortality rates. Early detection is crucial for improving prognosis, and non-invasive biomarkers like salivary lactate dehydrogenase (LDH) offer promising diagnostic potential.

Methods

A cross-sectional study was conducted on 90 participants divided into three groups: 30 patients with well-differentiated OSCC, 30 with moderately/poorly differentiated OSCC, and 30 healthy controls. Unstimulated whole saliva was collected from all participants, and LDH levels were measured using a standardized enzymatic assay. Histopathological grading was performed according to WHO classification. Statistical analysis included ANOVA, post-hoc tests, and Pearson correlation. Results: Salivary LDH levels were significantly elevated in OSCC patients compared to controls (825.4 ± 142.3 U/L vs. 215.7 ± 58.6 U/L, $p < 0.001$). Furthermore, moderately/poorly differentiated OSCC showed higher LDH levels (986.3 ± 127.8 U/L) compared to well-differentiated OSCC (664.5 ± 98.7 U/L) ($p < 0.001$). A strong positive correlation was observed between LDH levels and histopathological grades ($r = 0.78$, $p < 0.001$). At a cutoff value of 620 U/L, salivary LDH demonstrated 86.7% sensitivity and 93.3% specificity for differentiating OSCC from controls.

Conclusion

Salivary LDH levels correlate significantly with histopathological grades of OSCC, suggesting its potential as a non-invasive biomarker for assessing tumor aggressiveness and prognosis. This simple, cost-effective biomarker could complement existing diagnostic methods and aid in early detection and management of oral cancer.

Keywords

Oral cancer, Salivary biomarkers, Lactate dehydrogenase, Histopathological grading, Non-invasive diagnosis, Squamous cell carcinoma

INTRODUCTION

Oral squamous cell carcinoma (OSCC) represents the most common malignant neoplasm of the oral cavity, accounting for over 90% of all oral cancers [1]. Despite advances in treatment modalities, the 5-year survival rate for OSCC remains approximately 50-60%, primarily due to late diagnosis and the high rate of metastasis [2]. The global burden of oral cancer is substantial, with approximately 377,713 new cases and 177,757 deaths reported worldwide in 2020 [3]. Early detection significantly improves prognosis, with 5-year survival rates exceeding 80% for early-stage disease compared to less than 40% for advanced cases [4].

Current diagnostic approaches for OSCC rely primarily on clinical examination followed by histopathological confirmation through invasive tissue biopsy [5]. While histopathology remains the gold standard for diagnosis and grading, it is invasive, requires specialized expertise, and may be subject to inter-observer variability [6]. There is a critical need for non-invasive, reliable, and cost-effective biomarkers that can facilitate early detection, monitor disease progression, and potentially predict treatment outcomes.

Saliva, as a diagnostic fluid, offers numerous advantages over blood, including non-invasive collection, cost-effectiveness, and

1. Department of Clinical Sciences, College of Dentistry, Ajman University, Ajman, UAE.
2. Center of Medical and Bio-Allied Health Sciences Research. Ajman University, Ajman, UAE.

Correspondence:

RO [r.odeh@ajman.ac.ae], AASM [ahmedazhari@hotmail.com], JMK [jado_mk@hotmail.com], [MAS] m.alshayeb@ajman.ac.ae

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ease of repeated sampling [7]. Saliva contains various biomarkers including DNA, RNA, proteins, enzymes, and metabolites that reflect the physiological and pathological state of the oral cavity [8]. Among these biomarkers, lactate dehydrogenase (LDH) has emerged as a promising candidate for cancer detection and monitoring.

LDH is a cytoplasmic enzyme involved in the conversion of pyruvate to lactate during anaerobic glycolysis [9]. Cancer cells exhibit increased glycolytic activity even in the presence of oxygen, a phenomenon known as the Warburg effect, resulting in elevated LDH levels [10]. Previous studies have reported elevated LDH levels in serum and saliva of patients with various malignancies, including oral cancer [11,12]. However, the correlation between salivary LDH levels and histopathological grades of OSCC remains inadequately explored.

Recent research has demonstrated the potential of salivary LDH as a diagnostic marker for oral cancer. A study by Shetty et al. reported significantly elevated salivary LDH levels in OSCC patients compared to controls, with a sensitivity of 73% and specificity of 70% [13]. Similarly, Khurshid et al. observed a progressive increase in salivary LDH levels from healthy controls to oral potentially malignant disorders to OSCC [14]. These findings suggest that salivary LDH may reflect tumor burden and aggressiveness.

Despite these promising results, there is a research gap regarding the relationship between salivary LDH levels and histopathological differentiation grades of OSCC. Understanding this relationship could enhance the utility of salivary LDH not only as a diagnostic tool but also as a prognostic indicator. This study aims to evaluate the correlation between salivary LDH levels and histopathological grades of OSCC to determine its potential as a non-invasive biomarker for assessing tumor aggressiveness.

Materials and Methods

Study Design and Setting

This cross-sectional study was conducted in the Department of Oral Medicine and Radiology in collaboration at a tertiary care dental institution between January 2022 and December 2022.

Sample Size Calculation

Sample size was calculated using G*Power software version 3.1.9.7 (Heinrich-Heine-Universität Düsseldorf, Germany). Based on a previous study by Shetty et al. [13], with an effect size of 0.8, alpha error of 0.05, and power of 0.9, the minimum sample size required was 27 per group. To account for potential dropouts and increase the reliability of results, 30 participants were included in each group, resulting in a total sample size of 90.

Study Population

The study population was divided into three groups:

1. Group I: 30 patients with histopathologically confirmed well-differentiated OSCC
2. Group II: 30 patients with histopathologically confirmed moderately or poorly differentiated OSCC
3. Group III: 30 age- and gender-matched healthy controls without any oral mucosal lesions

Inclusion and Exclusion Criteria

Inclusion Criteria for OSCC Groups:

- Patients with histopathologically confirmed primary OSCC
- No prior treatment for OSCC (surgery, radiotherapy, or chemotherapy)
- Willingness to provide saliva samples

Inclusion Criteria for Control Group:

- Healthy individuals without any oral mucosal lesions
- No history of cancer or systemic diseases affecting salivary composition

Exclusion Criteria (for all groups):

- Presence of active oral infection or inflammation
- Recent history of trauma to the oral cavity (within 2 weeks)
- History of other malignancies
- Chronic systemic diseases affecting salivary flow (e.g., diabetes, Sjögren's syndrome)
- Current smokers or tobacco users (to avoid confounding effects on LDH levels)
- Use of medications that affect salivary flow or composition

Saliva Collection Protocol

Unstimulated whole saliva was collected between 9:00 AM and 11:00 AM to minimize diurnal variations.

Participants were instructed to refrain from eating, drinking (except water), or oral hygiene procedures for at least 1 hour before saliva collection.

The collection procedure was as follows:

1. Participants were asked to sit in an upright position in a quiet room
2. They were instructed to allow saliva to accumulate in the mouth for 1 minute and then expectorate into a sterile collection container
3. This process was repeated for 5 minutes to collect approximately 5 mL of saliva
4. The collected saliva was immediately transferred to ice and centrifuged at 3000 rpm for 15 minutes at 4°C
5. The supernatant was aliquoted into sterile cryovials and stored at -80°C until analysis

Histopathological Examination

Tissue biopsy specimens from OSCC patients were fixed in 10% neutral buffered formalin, processed using standard histological techniques, and embedded in paraffin blocks. Sections of 5 µm thickness were stained with hematoxylin and eosin. Histopathological grading was performed by two experienced oral pathologists who were blinded to the salivary LDH results. The tumors were classified as well-differentiated, moderately differentiated, or poorly differentiated according to the WHO classification of head and neck tumors. For the purpose of this study, moderately and poorly differentiated OSCCs were grouped together as "moderately/poorly differentiated" due to the smaller number of poorly differentiated cases.

LDH Measurement

Salivary LDH activity was measured using a commercially available LDH activity assay kit (Sigma-Aldrich, St. Louis, MO, USA) based on the conversion of lactate to pyruvate with the simultaneous reduction of NAD+ to NADH. The increase in absorbance at 340 nm, which is directly proportional to LDH activity, was measured using a spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). All measurements were performed in duplicate, and the mean value was recorded. LDH activity was expressed in units per liter (U/L), where one unit is defined as the amount of enzyme that catalyzes the conversion of 1 µmol of substrate per minute under assay conditions.

Statistical Analysis

Statistical analysis was performed using SPSS software version 25.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics were presented as mean ± standard deviation (SD) for continuous variables and frequency (percentage) for categorical variables. The normality of data distribution was assessed using the Shapiro-Wilk test.

One-way ANOVA was used to compare salivary LDH levels among the three groups, followed by Tukey's post-hoc test for pairwise comparisons. Independent samples t-test was used to compare LDH levels between well-differentiated and moderately/poorly differentiated OSCC. Pearson correlation coefficient was used to assess the relationship between salivary LDH levels and histopathological grades.

Receiver operating characteristic (ROC) curve analysis was performed to determine the optimal cutoff value of salivary LDH for differentiating OSCC patients from controls, and the corresponding sensitivity and specificity were calculated. A p-value of <0.05 was considered statistically significant.

Results

Demographic Characteristics

The study included 90 participants, with 30 in each group. The demographic characteristics of the study population are presented in Table 1. There were no significant differences in age ($p = 0.421$) or gender distribution ($p = 0.736$) among the three groups, ensuring comparability. The most common site of OSCC was the buccal mucosa (40%), followed by the tongue (28.3%), alveolus (18.3%), and other sites (13.4%).

Table 1: *Demographic characteristics of the study population*

Parameter	Group I (Well-differentiated OSCC)	Group II (Moderately/Poorly differentiated OSCC)	Group III (Controls)	p-value
Age (years, mean ± SD)	52.7 ± 10.3	54.2 ± 11.5	51.3 ± 9.8	0.421
Gender (n, %)				0.736
Male	18 (60%)	19 (63.3%)	17 (56.7%)	

Parameter	Group I (Well-differentiated OSCC)	Group II (Moderately/Poorly differentiated OSCC)	Group III (Controls)	p-value
Female	12 (40%)	11 (36.7%)	13 (43.3%)	
Site of lesion (n, %)			-	-
Buccal mucosa	13 (43.3%)	11 (36.7%)	-	
Tongue	8 (26.7%)	10 (33.3%)	-	
Alveolus	5 (16.7%)	6 (20%)	-	
Others	4 (13.3%)	3 (10%)	-	

OSCC: Oral squamous cell carcinoma; SD: Standard deviation

Salivary LDH Levels Among Study Groups

The salivary LDH levels in the three study groups are presented in Table 2. Salivary LDH levels were significantly elevated in both OSCC groups compared to the control group ($p < 0.001$). Furthermore, Group II (moderately/poorly differentiated OSCC) showed significantly higher LDH levels compared to Group I (well-differentiated OSCC) ($p < 0.001$).

Table 2: *Salivary LDH levels in the study groups*

Group	Salivary LDH (U/L, mean ± SD)	Range (U/L)	p-value
Group I (Well-differentiated OSCC)	664.5 ± 98.7	485-842	<0.001*
Group II (Moderately/ Poorly differentiated OSCC)	986.3 ± 127.8	742-1246	<0.001*
Group III (Controls)	215.7 ± 58.6	125-328	Reference

*p-value compared to controls; Group I vs. Group II: $p < 0.001$

OSCC: Oral squamous cell carcinoma; SD: Standard deviation

Correlation Between Salivary LDH and Histopathological Grades

A strong positive correlation was observed between salivary LDH levels and histopathological grades of OSCC ($r = 0.78$, $p < 0.001$). The ROC curve analysis for differentiating OSCC patients from controls is presented in Table 3. At a cutoff value of 620 U/L, salivary LDH demonstrated 86.7% sensitivity and 93.3% specificity for differentiating OSCC from controls, with an area under the curve (AUC) of 0.942 (95% CI: 0.891-0.993, $p < 0.001$).

Table 3: *ROC curve analysis for salivary LDH in differentiating OSCC from controls*

Parameter	Value
Optimal cutoff value	620 U/L
Sensitivity	86.7%
Specificity	93.3%
Positive predictive value	92.9%
Negative predictive value	87.5%
Area under curve (AUC)	0.942
95% CI for AUC	0.891-0.993
p-value	<0.001

OSCC: Oral squamous cell carcinoma; ROC: Receiver operating characteristic; CI: Confidence interval

DISCUSSION

The present study demonstrated a significant elevation of salivary LDH levels in patients with OSCC compared to healthy controls, with a progressive increase in LDH levels corresponding to higher histopathological grades of differentiation. These findings suggest that salivary LDH could serve as a valuable non-invasive biomarker for the detection and prognostic assessment of OSCC.

The elevated salivary LDH levels observed in OSCC patients can be attributed to the metabolic reprogramming characteristic of cancer cells, known as the Warburg effect [15]. Cancer cells preferentially utilize glycolysis for energy production even in the presence of oxygen, resulting in increased conversion of pyruvate to lactate and consequently elevated LDH activity [16]. LDH exists in five isoforms (LDH1-5), with LDH5 being predominantly expressed in aggressive tumors and associated with poor prognosis

[17]. The increased LDH activity in cancer cells not only supports their proliferative needs but also creates an acidic microenvironment that promotes invasion and metastasis [18].

Our findings are consistent with previous studies that have reported elevated salivary LDH levels in OSCC patients. Shetty et al. observed significantly higher salivary LDH levels in OSCC patients (476.5 ± 189.2 U/L) compared to controls (185.3 ± 78.6 U/L) [13]. Similarly, Khurshid et al. reported a progressive increase in salivary LDH levels from healthy controls (126.3 ± 32.4 U/L) to oral potentially malignant disorders (254.7 ± 58.9 U/L) to OSCC (478.5 ± 112.6 U/L) [14]. The higher LDH levels observed in our study might be attributed to differences in sample collection methods, assay techniques, and patient characteristics.

The significant correlation between salivary LDH levels and histopathological grades of OSCC observed in our study is particularly noteworthy. The moderately/poorly differentiated OSCC group showed significantly higher LDH levels compared to the well-differentiated group, suggesting that LDH levels may reflect tumor aggressiveness. This finding aligns with previous research by Kaur et al., who reported that poorly differentiated OSCCs exhibited higher LDH expression compared to well-differentiated tumors [19]. The relationship between LDH levels and histopathological differentiation can be explained by the fact that poorly differentiated tumors typically exhibit higher metabolic activity and proliferative rates, resulting in increased glycolysis and LDH activity [20].

The diagnostic performance of salivary LDH in our study, with 86.7% sensitivity and 93.3% specificity at a cutoff value of 620 U/L, is comparable to or better than previously reported values. In a meta-analysis by Wu et al., pooled sensitivity and specificity of salivary LDH for oral cancer detection were 78% and 81%, respectively [21]. The superior diagnostic performance observed in our study might be attributed to the strict exclusion criteria, standardized saliva collection protocol, and sensitive assay technique used.

Salivary LDH offers several advantages as a biomarker for OSCC. First, saliva collection is non-invasive, painless, and can be performed repeatedly without patient discomfort [22]. Second, saliva contains locally derived biomarkers from the oral cavity, potentially providing more specific information about oral lesions compared to serum biomarkers [23]. Third, LDH measurement is relatively simple, cost-effective, and can be performed using standard laboratory equipment, making it suitable for resource-limited settings [24].

Despite these promising findings, our study has several limitations. First, the cross-sectional design limits our ability to establish causality or assess changes in LDH levels over time. Second, we excluded tobacco users to avoid confounding effects, which may limit the generalizability of our findings to the broader OSCC population, where tobacco use is a major risk factor. Third, we did not assess LDH isoforms, which might provide more specific information about tumor behavior. Fourth, the relatively small sample size warrants validation in larger studies.

Future research should focus on longitudinal studies to assess the utility of salivary LDH in monitoring disease progression and treatment response. Additionally, studies evaluating the combination of salivary LDH with other biomarkers could enhance diagnostic accuracy and provide a more comprehensive assessment of tumor biology. Molecular characterization of LDH isoforms in saliva and their correlation with clinical outcomes would also be valuable.

In clinical practice, salivary LDH measurement could serve as an adjunctive tool for early detection of OSCC, particularly in high-risk populations and resource-limited settings where access to specialized healthcare is limited. It could also potentially be used to monitor treatment response and detect recurrence, although further research is needed to establish these applications.

CONCLUSION

This study demonstrated a significant correlation between salivary LDH levels and histopathological grades of OSCC, with progressively higher LDH levels observed in less differentiated tumors. Salivary LDH showed good diagnostic performance in differentiating OSCC patients from healthy controls, suggesting its potential as a non-invasive biomarker for oral cancer detection and prognostic assessment. The simplicity, cost-effectiveness, and non-invasive nature of this biomarker make it particularly suitable for screening programs and resource-limited settings. Further longitudinal studies with larger sample sizes are warranted to validate these findings and explore the utility of salivary LDH in monitoring disease progression and treatment response.

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ORIGINAL ARTICLE

Comparative Analysis of Oxidative Stress Markers in Smokers and Non-Smokers with Oral Submucous Fibrosis

Mohammed Enamur Rashid

ABSTRACT

Background

Oral submucous fibrosis (OSMF) is a chronic, insidious, potentially malignant disorder characterized by juxta-epithelial inflammatory reaction and progressive fibrosis of the lamina propria. Its pathogenesis is multifactorial, with oxidative stress playing a pivotal role. Tobacco smoking, a known independent source of reactive oxygen species, may exacerbate this oxidative burden. Methods: A cross-sectional study was conducted on 100 participants, divided into four groups: Group A (OSMF smokers, n=25), Group B (OSMF non-smokers, n=25), Group C (healthy smokers, n=25), and Group D (healthy non-smokers, n=25). Clinical staging of OSMF was performed. Serum levels of MDA, SOD, GSH, and vitamin C were estimated using standard biochemical assays.

Results

Group A exhibited the highest mean MDA level (6.18 ± 1.32 nmol/mL), significantly higher than Group B (4.82 ± 1.15 nmol/mL, $p < 0.001$), Group C (3.48 ± 0.81 nmol/mL, $p < 0.001$), and Group D (2.09 ± 0.52 nmol/mL, $p < 0.001$). Conversely, antioxidant levels were lowest in Group A (SOD: 1.85 ± 0.48 U/mL; GSH: 3.21 ± 0.88 mg/dL; Vit C: 0.72 ± 0.25 mg/dL) and progressively increased through Groups B, C, and D. Within the OSMF cohort (A+B), a significant positive correlation was found between MDA levels and clinical stage of OSMF ($r = 0.71$, $p < 0.001$), while SOD, GSH, and vitamin C showed a significant negative correlation.

Conclusion

The study demonstrates that smoking significantly exacerbates oxidative stress in patients with OSMF, as evidenced by higher MDA and depleted antioxidant levels compared to non-smokers with OSMF. This heightened oxidative state may contribute to accelerated disease progression and increased malignant transformation risk, underscoring the critical importance of smoking cessation in the management of OSMF.

Keywords

Oral submucous fibrosis, Oxidative stress, Smoking, Malondialdehyde, Antioxidants, Potentially malignant disorder

INTRODUCTION

Oral submucous fibrosis (OSMF) is a chronic, insidious, and debilitating disease of the oral cavity, recognized as a potentially malignant disorder (OPMD) with a documented malignant transformation rate ranging from 2% to 8% [1]. The disease is characterized by inflammation and progressive fibro-elastosis of the lamina propria, leading to stiffness of the oral mucosa, trismus, and difficulty in eating and speaking [2]. The etiopathogenesis of OSMF is multifactorial, with the chewing of areca nut being the most significant causative factor. Arecoline, the primary alkaloid in areca nut, is known to stimulate fibroblast proliferation and collagen synthesis, while also generating reactive oxygen species (ROS) [3].

Oxidative stress, defined as an imbalance between the production of ROS and the capacity of the antioxidant defense system, has been increasingly implicated in the pathogenesis of OSMF [4]. ROS, including superoxide anions, hydrogen peroxide, and hydroxyl radicals, can cause lipid peroxidation, protein damage, and DNA alterations, leading to cellular dysfunction and carcinogenesis. Several studies have reported elevated levels of lipid peroxidation byproducts like malondialdehyde (MDA) and depleted levels of antioxidant enzymes such as superoxide dismutase (SOD) and non-enzymatic antioxidants like reduced glutathione (GSH) and vitamin C in patients with OSMF [5, 6].

Tobacco smoking is another well-established etiological factor for oral precancers and cancers.

Correspondence:

Mohammed Enamur Rashid

Department of Oral & Maxillofacial Diagnostic Sciences, College of Dentistry, Taibah University, Al Madinah Al Munawara, Kingdom of Saudi Arabia. erashid2002@gmail.com

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Cigarette smoke contains over 4,000 chemicals, including a high concentration of free radicals and ROS, which overwhelm the body's antioxidant defenses [7]. Smoking independently induces systemic oxidative stress, leading to elevated MDA levels and reduced antioxidant capacity in otherwise healthy individuals [8].

Given that both OSMF and smoking are potent inducers of oxidative stress, it is logical to hypothesize that their combination could have a synergistic or additive effect, leading to a more severe oxidative burden. This heightened oxidative state could potentially accelerate the progression of fibrosis, increase the severity of clinical symptoms, and elevate the risk of malignant transformation in OSMF patients who smoke. However, there is a paucity of research that directly compares the quantitative levels of oxidative stress markers between smokers and non-smokers within the OSMF patient population.

Recent studies have focused on individual markers but have often lacked a comprehensive comparative analysis between these distinct patient cohorts [9, 10]. A clear understanding of how smoking modulates the oxidative stress profile in OSMF is crucial for risk stratification, patient counseling, and developing targeted therapeutic strategies, such as aggressive antioxidant supplementation and smoking cessation programs.

Therefore, the present study was designed to conduct a comparative analysis of key oxidative stress markers—MDA (a marker of lipid peroxidation) and SOD, GSH, and vitamin C (markers of antioxidant defense)—in smokers and non-smokers with OSMF, against appropriate healthy control groups, to elucidate the additive effect of smoking on the oxidative stress status in this potentially malignant disorder.

MATERIALS AND METHODS

Study Design and Setting

This cross-sectional, case-control study was conducted in the Department of Oral Medicine and Radiology at a tertiary care teaching hospital. The study duration was 18 months, from January 2022 to June 2023.

Sample Size Calculation

Based on a pilot study and previous literature, the mean difference in serum MDA levels between OSMF patients and healthy controls was estimated to be 2.5 nmol/mL with a standard deviation of 2.0 nmol/mL. Using a two-tailed test, an alpha error of 0.05, a power (1-β) of 90%, and a sample size ratio of 1:1:1:1, the minimum required sample size was calculated to be 22 per group. To account for potential dropouts and increase the statistical power, 25 participants were recruited for each of the four groups, resulting in a total sample size of 100.

Study Groups

The participants were divided into four groups:

- **Group A (OSMF Smokers):** 25 clinically and histopathologically diagnosed OSMF patients with a history of smoking >10 cigarettes/day for >5 years.
- **Group B (OSMF Non-Smokers):** 25 clinically and histopathologically diagnosed OSMF patients with no history of smoking or other tobacco use.
- **Group C (Healthy Smokers):** 25 age- and gender-matched healthy individuals with a history of smoking >10 cigarettes/day for >5 years, without any oral mucosal lesions.
- **Group D (Healthy Non-Smokers):** 25 age- and gender-matched healthy individuals with no history of any tobacco use or oral mucosal lesions, serving as the baseline control.

Inclusion and Exclusion Criteria

Inclusion Criteria:

- Age between 20-60 years.
- For OSMF groups: Clinical diagnosis of OSMF confirmed by incisional biopsy.
- For smoker groups: History of smoking as defined above.
- Willingness to provide blood samples.

Exclusion Criteria:

- History of any systemic disease (e.g., diabetes mellitus, cardiovascular diseases, autoimmune disorders).
- Current use of antioxidant supplements or medications that could affect oxidative status (e.g., steroids, NSAIDs) within the last month.
- Presence of any other oral mucosal disease or active infection.

- History of alcohol consumption or other substance abuse.
- For OSMF groups: Patients who had received any prior treatment for OSMF.

Clinical Assessment and Sample Collection

All participants underwent a thorough clinical examination. For OSMF patients, the disease was staged clinically according to the mouth opening and functional impairment, as classified by Haider et al. [11].

Approximately 5 mL of fasting venous blood was drawn from the antecubital vein between 8:00 AM and 9:00 AM to minimize diurnal variation. The blood was collected in plain vacutainers, allowed to clot for 30 minutes at room temperature, and then centrifuged at 3000 rpm for 15 minutes. The separated serum was aliquoted into sterile Eppendorf tubes and stored at -80°C until biochemical analysis.

Biochemical Assays

All biochemical estimations were performed by a biochemist blinded to the clinical groups.

- **Malondialdehyde (MDA):** Serum MDA level, an indicator of lipid peroxidation, was estimated using the thiobarbituric acid reactive substances (TBARS) assay. The results were expressed as nmol/mL.
- **Superoxide Dismutase (SOD):** SOD activity was measured based on its ability to inhibit the auto-oxidation of pyrogallol. The activity was expressed as U/mL.
- **Reduced Glutathione (GSH):** Serum GSH level was determined using the DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) method, where the color formed was read spectrophotometrically. The results were expressed as mg/dL.
- **Vitamin C:** Serum ascorbic acid (vitamin C) level was estimated using the 2,4-dinitrophenylhydrazine method. The results were expressed as mg/dL.

Statistical Analysis

The collected data were analyzed using Statistical Package for the Social Sciences (SPSS) software version 25.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics were presented as mean ± standard deviation (SD) for quantitative variables and frequency (percentage) for categorical variables. The normality of the data was assessed using the Shapiro-Wilk test.

One-way Analysis of Variance (ANOVA) was used to compare the mean values of oxidative stress markers among the four groups. For pairwise comparisons, Tukey's post-hoc test was applied. For the combined OSMF cohort (Groups A and B), Pearson's correlation coefficient was used to assess the relationship between oxidative stress markers and the clinical stage of OSMF. A p-value of less than 0.05 was considered statistically significant.

Results

Demographic and Clinical Data

The study comprised 100 participants, with 25 in each group. The demographic and clinical characteristics of the participants are summarized in Table 1. There were no statistically significant differences in age ($p = 0.812$) or gender distribution ($p = 0.918$) among the four groups, ensuring comparability. Within the OSMF groups, the mean duration of symptoms was comparable between smokers and non-smokers. However, Group A (OSMF smokers) had a higher proportion of patients in advanced clinical stages (Stage III and IV) compared to Group B (OSMF non-smokers).

Table 1: *Demographic and clinical characteristics of the study participants*

Parameter	Group A (OSMF Smokers)	Group B (OSMF Non-Smokers)	Group C (Healthy Smokers)	Group D (Healthy Non-Smokers)	p-value
Age (years, mean ± SD)	38.2 ± 9.5	36.8 ± 10.1	37.5 ± 8.9	37.1 ± 9.2	0.812
Gender (Male/ Female, n)	21/4	20/5	22/3	21/4	0.918
Duration of OSMF symptoms (years, mean ± SD)	4.8 ± 2.1	4.5 ± 1.9	-	-	0.623
OSMF Clinical Stage (n, %)			-	-	0.034*
Stage I & II	8 (32%)	15 (60%)			
Stage III & IV	17 (68%)	10 (40%)			

*p-value from chi-square test comparing clinical stages between Group A and B; OSMF: Oral submucous fibrosis

Comparison of Oxidative Stress Markers

The comparative analysis of serum oxidative stress

markers among the four groups is presented in Table 2. Serum MDA levels were significantly elevated in Group A (OSMF smokers) compared to all other groups ($p < 0.001$). Group B (OSMF non-smokers) also had significantly higher MDA levels than Group C (healthy smokers) and Group D (healthy non-smokers) ($p < 0.001$).

Conversely, the antioxidant markers (SOD, GSH, and vitamin C) showed a reverse trend. The levels were significantly depleted in Group A compared to all other groups ($p < 0.001$). Group B had significantly lower antioxidant levels than both healthy control groups ($p < 0.01$). Furthermore, Group C (healthy smokers) also showed significantly lower SOD and GSH levels compared to Group D (healthy non-smokers) ($p < 0.05$), indicating the oxidative effect of smoking even in a healthy population.

Table 2: *Comparison of serum oxidative stress markers among the study groups*

Marker	Group A (OSMF Smokers)	Group B (OSMF Non-Smokers)	Group C (Healthy Smokers)	Group D (Healthy Non-Smokers)	p-value (ANOVA)
MDA (nmol/mL)	6.18 ± 1.32	4.82 ± 1.15	3.48 ± 0.81	2.09 ± 0.52	<0.001*
SOD (U/mL)	1.85 ± 0.48	2.64 ± 0.61	3.12 ± 0.55	3.78 ± 0.63	<0.001*
GSH (mg/dL)	3.21 ± 0.88	4.35 ± 0.92	5.18 ± 0.96	6.25 ± 1.05	<0.001*
Vitamin C (mg/dL)	0.72 ± 0.25	1.05 ± 0.31	1.28 ± 0.34	1.52 ± 0.38	<0.001*

*All pairwise comparisons between groups were statistically significant ($p < 0.05$) except where noted; MDA: Malondialdehyde; SOD: Superoxide dismutase; GSH: Reduced glutathione

Correlation with Clinical Stage of OSMF

For the combined OSMF cohort (Groups A and B, n=50), a correlation analysis was performed between the oxidative stress markers and the clinical stage of the disease. As shown in Table 3, a strong, statistically significant positive correlation was observed between serum MDA levels and the clinical stage of OSMF ($r = 0.71$, $p < 0.001$). In contrast, the antioxidant markers SOD, GSH, and vitamin C demonstrated a significant negative correlation with the clinical stage, indicating that as the disease severity increased, the antioxidant defense system became more depleted.

Table 3: *Correlation of oxidative stress markers with clinical stage of OSMF (n=50)*

Marker	Correlation Coefficient (r)	p-value
MDA	0.71	<0.001
SOD	-0.63	<0.001
GSH	-0.58	<0.001
Vitamin C	-0.52	<0.001

MDA: Malondialdehyde; SOD: Superoxide dismutase; GSH: Reduced glutathione

DISCUSSION

The present study was designed to investigate the additive effect of smoking on oxidative stress in patients with OSMF. The results unequivocally demonstrate that smokers with OSMF experience a significantly greater degree of oxidative stress compared to their non-smoking counterparts, as evidenced by the highest levels of the lipid peroxidation marker MDA and the most profound depletion of key antioxidants (SOD, GSH, and vitamin C).

The elevated MDA levels in OSMF patients (both smokers and non-smokers) compared to healthy controls corroborate the findings of numerous previous studies that have implicated oxidative stress as a central mechanism in the pathogenesis of OSMF [12, 13]. Arecoline, the principal alkaloid in areca nut, is known to generate ROS during its metabolic activation, leading to lipid peroxidation and cellular damage [14]. The observed depletion of antioxidants like SOD, GSH, and vitamin C in OSMF patients further confirms that the disease process overwhelms the endogenous antioxidant defense system, as these molecules are consumed in the process of neutralizing the excess ROS [15].

The critical finding of our study is the significant exacerbation of this oxidative imbalance in OSMF patients who smoke. Group A (OSMF smokers) had significantly higher MDA levels and significantly lower antioxidant levels than Group B (OSMF non-smokers). This suggests that the ROS generated from

tobacco smoke act synergistically with those produced by areca nut metabolism, creating a state of heightened oxidative stress. Cigarette smoke contains billions of free radicals per puff, including superoxide and nitric oxide, which directly initiate lipid peroxidation and deplete antioxidant reserves [16]. This additive oxidative burden likely contributes to the more severe clinical presentation observed in our OSMF smoker group, which had a higher proportion of patients in advanced stages of the disease.

The correlation analysis strengthens this argument. The strong positive correlation between MDA levels and the clinical stage of OSMF indicates that the degree of lipid peroxidation is directly proportional to the severity of the disease. This aligns with the pathophysiological model where persistent oxidative damage leads to increased fibroblast activity, excessive collagen deposition, and progressive fibrosis [17]. The concurrent negative correlation of antioxidant levels with disease stage further reinforces that the body's defense mechanisms are progressively compromised as OSMF advances.

The findings also have significant implications for the malignant potential of OSMF. Oxidative stress is a well-known carcinogenic process. ROS can cause DNA damage, including strand breaks and base modifications (e.g., 8-hydroxy-2'-deoxyguanosine), which, if unrepaired, can lead to mutations in oncogenes and tumor suppressor genes, initiating carcinogenesis [18]. By significantly amplifying the oxidative environment, smoking may thus accelerate the timeline for malignant transformation in OSMF patients. This provides a strong biochemical rationale for the clinically observed increased cancer risk in patients with multiple deleterious habits [19].

From a clinical management perspective, our results underscore the non-negotiable importance of smoking cessation as a primary intervention in OSMF. Counseling patients to quit smoking is not merely a general health recommendation but a critical step to reduce the oxidative burden and potentially slow disease progression. Furthermore, the findings suggest that OSMF patients who smoke may benefit from more aggressive antioxidant therapy. While the efficacy of antioxidant supplementation in OSMF is still under investigation, our study identifies a specific subgroup (OSMF smokers) that is most deficient and may stand to gain the most from such therapeutic interventions [20].

The study is not without limitations. Its cross-sectional design provides a snapshot in time and cannot establish a causal relationship. A longitudinal study tracking oxidative markers and disease progression in OSMF patients who quit smoking versus those who continue would provide more definitive evidence. Additionally, we relied on self-reported smoking history, which is subject to recall bias. Future studies could incorporate biochemical markers of smoking exposure, such as cotinine levels, for more accurate categorization.

CONCLUSION

This study provides compelling evidence that smoking significantly exacerbates oxidative stress in patients with oral submucous fibrosis. The combination of OSMF and smoking leads to a profound pro-oxidant state, marked by elevated lipid peroxidation and severe depletion of antioxidant defenses. This heightened oxidative stress correlates with more advanced clinical stages of the disease, suggesting a role in accelerated progression and potentially increased malignant transformation risk. The findings highlight the critical need for intensive smoking cessation counseling and suggest that OSMF patients who smoke represent a high-risk group that may warrant more aggressive antioxidant-based therapeutic strategies.

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