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Salivary oxidative stress biomarkers across oral submucous fibrosis stages among Indian patients

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Abstract:

Oral submucous fibrosis is a chronic potentially malignant disorder in which clinical staging is mainly based on mouth opening and fibrotic bands, while non-invasive biochemical indicators of disease severity remain insufficiently used. Therefore, it is of interest to evaluate the salivary malondialdehyde, 8-hydroxy-2-deoxyguanosine, superoxide dismutase, catalase and total antioxidant capacity in 120 participants, including 80 clinically staged oral submucous fibrosis patients and 40 healthy controls. Unstimulated whole saliva was collected under standardized morning conditions, biomarkers were measured by enzyme-linked and colorimetric assays and clinical staging was performed using interincisal mouth opening, burning sensation score and palpable fibrotic band distribution. Mean salivary malondialdehyde increased from 2.18 ± 0.64 micromol/L in controls to 6.41 ± 1.32 micromol/L in stage III disease, while total antioxidant capacity declined from 1.42 ± 0.31 to 0.72 ± 0.22 mmol/L; oxidative markers correlated negatively with mouth opening and positively with clinical stage ($p < 0.001$). Thus, data shows that a salivary oxidative stress panel can provide a practical adjunct for staging and monitoring oral submucous fibrosis.

Keywords: Oral submucous fibrosis, saliva, malondialdehyde, 8-OHdG, antioxidant capacity, clinical staging

Background:

Oral submucous fibrosis is a chronic, progressive, fibrotic disorder of the oral mucosa with recognized malignant potential and a strong relationship with areca nut exposure [1]. The condition is characterized by juxta-epithelial inflammation, epithelial atrophy, collagen deposition, progressive limitation of mouth opening and persistent burning sensation. It affects quality of life by interfering with speech, eating, oral hygiene, prosthetic rehabilitation and clinical examination. Its public health importance is heightened in regions where commercially prepared areca nut products are widely available and consumed at younger ages [2]. The clinical course of oral submucous fibrosis is not uniform. Some individuals remain stable with mild mucosal stiffness, whereas others develop rapid progression, severe trismus, mucosal ulceration and epithelial dysplasia. Clinical staging based on mouth opening and palpable bands is useful for routine practice, but it does not fully capture molecular stress, epithelial damage, or malignant transformation risk [3]. A non-invasive biomarker panel may therefore help clinicians identify patients with active oxidative injury even before severe restriction is obvious. Oxidative stress is considered one of the key biological pathways linking areca nut exposure to mucosal fibrosis and epithelial instability. Arecoline and other areca alkaloids can stimulate fibroblasts, alter collagen metabolism and increase reactive oxygen species. Reactive species may damage membrane lipids, proteins and DNA, while antioxidant defenses may be consumed during chronic exposure [4]. This imbalance is clinically relevant because oxidative DNA damage and impaired antioxidant

response can create a microenvironment favorable for dysplasia and malignant change [5]. Saliva is an attractive diagnostic medium because it is easy to collect, non-invasive, inexpensive and directly bathes the affected mucosa. Salivary malondialdehyde reflects lipid peroxidation, while 8-hydroxy-2-deoxyguanosine reflects oxidative DNA damage. Enzymatic antioxidants such as superoxide dismutase and catalase indicate protective response, whereas total antioxidant capacity provides an integrated estimate of salivary defense reserve [6]. Several studies have reported altered salivary oxidative markers in oral potentially malignant disorders, but the degree to which a combined marker panel correlates with clinical staging of oral submucous fibrosis requires further evaluation [7]. Current diagnostic practice depends heavily on visual and tactile assessment. Although biopsy remains important when dysplasia is suspected, routine repeated biopsy is not practical for every patient at every visit. Salivary biomarkers may complement clinical examination by providing objective biochemical evidence of disease activity [8]. The literature suggests that salivary oxidative stress increases with clinical severity, yet there is variability in sample size, staging criteria, biomarker selection and assay methods. These differences limit direct translation into chairside risk stratification [9]. Therefore, it is of interest to evaluate the salivary oxidative stress biomarkers in oral submucous fibrosis and correlate them with clinical stage and disease severity.

Materials and Methods:

This observational case-control study was conducted in the outpatient dental setting over 12 months. A total of 120 participants aged 18 to 60 years were recruited after written informed consent. The study group included 80 clinically diagnosed oral submucous fibrosis patients, while the control group included 40 age- and sex-matched healthy individuals without oral mucosal lesions or areca nut habit. The case group was subdivided into stage I, stage II and stage III according to interincisal mouth opening, burning sensation and distribution of palpable fibrotic bands. Inclusion criteria for the case group were clinically evident oral submucous fibrosis, history of areca nut or related product use and willingness to undergo salivary analysis. Exclusion criteria were current systemic antioxidant therapy, oral cancer, previous treatment for oral submucous fibrosis within three months, diabetes mellitus, chronic kidney or liver disease, autoimmune disorders, active oral infection, pregnancy, smoking without areca exposure and inability to provide an adequate saliva sample. Controls were excluded if they had any tobacco or areca habit, oral mucosal lesions, or systemic inflammatory disease. Clinical examination was performed by two calibrated examiners. Interincisal mouth opening was measured using a digital Vernier caliper as the distance between the upper and lower central incisal edges at maximum unassisted opening. Burning sensation was recorded using a 10-point visual analogue scale after exposure to spicy stimulus history. Fibrotic bands were palpated in the buccal mucosa, labial mucosa, soft palate and retro molar area. Clinical staging was assigned after consensus review. Unstimulated whole saliva was collected between 9:00 AM and 11:00 AM. Participants were asked to avoid food, beverages, oral hygiene procedures and chewing products for at least 90 minutes before collection. Saliva was collected by passive drooling into sterile tubes for five minutes, centrifuged at 3000 rpm for 10 minutes and stored at -80 degrees C until analysis. Malondialdehyde was estimated by thiobarbituric acid reactive substances assay. 8-hydroxy-2-deoxyguanosine was estimated using enzyme-linked immunosorbent assay. Superoxide dismutase, catalase and total antioxidant capacity were measured using commercially available colorimetric kits according to manufacturer instructions. Data were entered into Microsoft Excel and

analyzed using SPSS version 26. Continuous variables were expressed as mean $\pm$ standard deviation, while categorical variables were expressed as frequency and percentage. Intergroup comparisons were performed using one-way ANOVA with Bonferroni post hoc testing. Categorical variables were compared using chi-square test. Pearson or Spearman correlation was used based on data distribution. Multivariable linear regression was performed to evaluate independent associations between clinical stage and biomarker levels after adjusting for age, sex, duration of habit and frequency of habit. A p value less than 0.05 was considered statistically significant.

Results:

Among 120 participants, the mean age was 36.8 ± 9.4 years in the oral submucous fibrosis group and 35.9 ± 8.7 years in controls. Males constituted 71.2% of cases and 67.5% of controls. The case group included 24 stage I, 31 stage II and 25 stage III patients. Mean mouth opening decreased progressively from 34.7 ± 3.2 mm in stage I to 19.8 ± 4.1 mm in stage III. Burning sensation scores and number of involved anatomical sites increased with advancing stage. Salivary oxidative stress markers differed significantly across groups. Mean malondialdehyde was 2.18 ± 0.64 micromol/L in controls, 3.68 ± 0.82 micromol/L in stage I, 4.92 ± 1.04 micromol/L in stage II and 6.41 ± 1.32 micromol/L in stage III ($p < 0.001$). Mean 8-hydroxy-2-deoxyguanosine showed a similar progressive increase. Antioxidant markers declined significantly with severity; total antioxidant capacity was 1.42 ± 0.31 mmol/L in controls and 0.72 ± 0.22 mmol/L in stage III patients ($p < 0.001$). Clinical stage showed a strong positive correlation with malondialdehyde ($r = 0.74$, $p < 0.001$) and 8-hydroxy-2-deoxyguanosine ($r = 0.70$, $p < 0.001$) and a strong negative correlation with total antioxidant capacity ($r = -0.68$, $p < 0.001$). Mouth opening correlated negatively with malondialdehyde ($r = -0.71$, $p < 0.001$) and positively with total antioxidant capacity ($r = 0.63$, $p < 0.001$). In adjusted regression, malondialdehyde and total antioxidant capacity remained independently associated with clinical stage. Demographic/clinical characteristics, salivary biomarker levels and correlation/regression analyses are summarized in **Tables 1, 2 and 3** respectively.

Table 1: Demographic and clinical characteristics of study participants

Variable	Controls (n=40)	Stage I (n=24)	Stage II (n=31)	Stage III (n=25)	p value
Age (years)	35.9 $\pm$ 8.7	34.8 $\pm$ 7.6	37.2 $\pm$ 8.9	38.5 $\pm$ 10.1	0.412
Male sex, n (%)	27 (67.5%)	16 (66.7%)	22 (71.0%)	19 (76.0%)	0.842
Habit duration (years)	0	4.8 $\pm$ 2.1	7.2 $\pm$ 3.4	9.6 $\pm$ 4.1	<0.001
Mouth opening (mm)	43.6 $\pm$ 4.2	34.7 $\pm$ 3.2	27.5 $\pm$ 3.8	19.8 $\pm$ 4.1	<0.001
Burning sensation VAS	0.4 $\pm$ 0.8	3.1 $\pm$ 1.2	5.2 $\pm$ 1.4	7.1 $\pm$ 1.3	<0.001

Table 2: Salivary oxidative stress and antioxidant biomarkers by clinical group

Biomarker	Controls	Stage I	Stage II	Stage III	p value
Malondialdehyde (micromol/L)	2.18 $\pm$ 0.64	3.68 $\pm$ 0.82	4.92 $\pm$ 1.04	6.41 $\pm$ 1.32	<0.001
8-OHdG (ng/mL)	1.94 $\pm$ 0.58	3.16 $\pm$ 0.74	4.28 $\pm$ 1.02	5.86 $\pm$ 1.35	<0.001
Superoxide dismutase (U/mL)	6.92 $\pm$ 1.28	5.81 $\pm$ 1.14	4.72 $\pm$ 1.07	3.88 $\pm$ 0.96	<0.001
Catalase (kU/L)	42.5 $\pm$ 8.1	35.9 $\pm$ 7.6	29.8 $\pm$ 6.4	24.1 $\pm$ 5.8	<0.001
Total antioxidant capacity (mmol/L)	1.42 $\pm$ 0.31	1.16 $\pm$ 0.28	0.91 $\pm$ 0.24	0.72 $\pm$ 0.22	<0.001

Table 3: Correlation and adjusted regression analysis of biomarkers with clinical severity

Parameter	Correlation with stage (r)	p value	Adjusted beta	95% CI
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Malondialdehyde	0.74	<0.001	0.48	0.36 to 0.61
8-OHdG	0.70	<0.001	0.39	0.25 to 0.54
Superoxide dismutase	-0.62	<0.001	-0.28	-0.41 to -0.13
Catalase	-0.59	<0.001	-0.24	-0.38 to -0.09
Total antioxidant capacity	-0.68	<0.001	-0.43	-0.57 to -0.29

Discussion:

The present study found a clear stage-wise increase in salivary oxidative damage markers and a parallel decrease in antioxidant defense among patients with oral submucous fibrosis. Malondialdehyde and 8-hydroxy-2-deoxyguanosine were lowest in healthy controls and highest in stage III disease, while superoxide dismutase, catalase and total antioxidant capacity declined with clinical severity. These findings support the biological model in which areca-related mucosal injury produces persistent oxidative stress that contributes to fibrosis and epithelial vulnerability [11]. The progressive rise in salivary malondialdehyde is clinically meaningful because lipid peroxidation is a downstream indicator of free radical-mediated membrane damage. The strong negative correlation between malondialdehyde and mouth opening suggests that oxidative stress is linked not only to epithelial injury but also to the fibrotic component of the disease. This observation agrees with previous work showing higher salivary lipid peroxidation in oral submucous fibrosis and oral potentially malignant disorders [12]. Increased 8-hydroxy-2-deoxyguanosine indicates oxidative DNA injury. This marker is relevant in oral submucous fibrosis because malignant transformation is a central concern. Although a single salivary value cannot diagnose dysplasia or cancer, elevated DNA damage markers may help identify patients requiring closer surveillance, habit cessation reinforcement and biopsy of suspicious sites. Biomarker-based follow-up may be particularly helpful when restricted mouth opening makes complete mucosal inspection difficult [13]. The decline in total antioxidant capacity and antioxidant enzymes reflects depletion of protective mechanisms. In chronic areca exposure, antioxidant defenses may initially increase as a compensatory response but later decline when oxidative burden becomes sustained. The present stage-wise decrease suggests that advanced disease is associated with insufficient salivary neutralizing capacity. This provides a rationale for studying antioxidant-based adjunctive strategies, although such interventions should not replace habit cessation and clinical monitoring [14]. The study also highlights the practical value of saliva as a diagnostic fluid. Salivary testing can be repeated without discomfort and may be integrated into follow-up visits. However, salivary biomarkers are influenced by hydration, circadian rhythm, periodontal inflammation, oral hygiene, diet and systemic conditions. For this reason, strict collection timing and exclusion criteria were applied. Any future chairside test should account for these biological and technical variables before being recommended for routine staging [15]. Clinical staging remains essential because biomarkers cannot replace examination. The strongest practical approach may be combined staging, in which mouth opening, fibrotic band extent, symptom score, habit profile and salivary oxidative panel are interpreted together. In the present dataset, malondialdehyde and total antioxidant capacity remained independently

associated with stage after adjustment for habit duration and frequency. This suggests that they may reflect disease activity beyond exposure history alone [16]. The results should be interpreted in view of certain limitations. The study was cross-sectional, so causality and temporal changes cannot be established. Histopathological grading was not performed in every case because the objective was non-invasive clinical staging. The biomarker panel was limited to select oxidative and antioxidant markers; inflammatory cytokines, collagen turnover markers and molecular dysplasia markers were not included. Longitudinal studies are needed to determine whether baseline biomarker levels predict progression, response to therapy, or malignant transformation [17]. Despite these limitations, the findings provide clinically useful evidence that salivary oxidative stress increases with oral submucous fibrosis severity. A simple panel combining malondialdehyde, 8-hydroxy-2-deoxyguanosine and total antioxidant capacity may offer a non-invasive adjunct to clinical staging. Future multicenter studies should define cutoff values, evaluate reproducibility across laboratories and test whether biomarker improvement follows habit cessation and medical management [18]. These findings should also be interpreted within the broader oral submucous fibrosis literature spanning clinical staging, natural history, malignant-risk surveillance and therapeutic evidence [10, 19-29]. Diagnostic adjuncts and newer molecular or biomarker studies likewise support multimodal assessment rather than replacement of clinical and histopathological evaluation [30-35]. A recent systematic review and meta-analysis similarly reported increased oxidative-stress markers with reduced antioxidant defenses in oral submucous fibrosis, supporting the direction of the present stage-wise findings [36].

Conclusion:

Salivary oxidative stress markers changed progressively with oral submucous fibrosis severity. Malondialdehyde and 8-OHdG increased, whereas antioxidant defenses decreased with advancing stage. These markers may complement clinical staging, but longitudinal validation is required before routine use.

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