

Estimation Of Copper, Zinc And Iron In Saliva And Serum And Its Correlation With Histological Grades Of Oral Submucous Fibrosis

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Abstract

INTRODUCTION Oral submucous fibrosis (OSMF) is a chronic, progressive disorder affecting the oral cavity, notably recognized for its potential to undergo malignant transformation. The condition typically begins with erythema, blister formation, and ulceration within the oral mucosa. Although its exact pathogenesis remains unclear, habitual betel quid chewing is regarded as the primary etiological factor. Although technological progress has been made, the application of inductively coupled plasma mass spectrometry (ICP-MS) in evaluating trace elements remains underexplored. This study highlights the necessity for continued research into these elements to facilitate early diagnosis and enhance the clinical management of oral submucous fibrosis (OSMF).

AIM & OBJECTIVE:

AIM To quantify, compare and correlate serum and salivary levels of copper, zinc and ferrum in patients with oral submucous fibrosis (OSMF) and healthy volunteers using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

OBJECTIVES

1. To quantify serum and salivary levels of copper, zinc and Ferrum in patients with OSMF (Group 1).
2. To quantify serum and salivary levels of copper, zinc and Ferrum in healthy volunteers (Group 2).
3. To compare and correlate serum and salivary levels of copper, zinc and Ferrum in different histopathological grades of OSMF.
4. To compare and correlate serum and salivary levels of copper, zinc and Ferrum between the groups.

MATERIALS AND METHODOLOGY-A case-control study involving 40 participants (20 OSMF patients, 20 healthy controls) was conducted at Teerthanker Mahaveer Dental College. Following clinical diagnosis, buccal mucosa punch biopsies were obtained under local anaesthesia, fixed in 10% formalin, sectioned into 4 µm slices, and mounted on albumin-coated slides.

For trace element analysis via ICP-MS, fasting venous blood and unstimulated saliva were collected, centrifuged, and stored at -40°C. Analytical procedures followed stringent calibration and quality control protocols to quantify copper, zinc, and iron—key biomarkers implicated in the pathophysiology and early detection of OSMF.

RESULTS:

Mean Age: Comparable between groups (35.75 vs. 37.15 years; $p = 0.7800$)

Gender Distribution: Male predominance in Group 1 (80%) vs. Group 2 (55%); not statistically significant ($p = 0.0910$)

Mouth Opening: Significantly reduced in Group 1 (21.80 ± 8.01 mm) vs. Group 2 (51.15 ± 6.69 mm); $p = 0.0001$

Regarding serum trace element levels Copper was significantly elevated in Group 1 (2799 ± 434.18 µg/l) compared to Group 2 (1906.30 ± 43.06 µg/l), $p = 0.0001$. Zinc was markedly lower in Group 1 (982.80 ± 212.35 µg/l) versus Group 2 (1655.95 ± 49.49 µg/l), $p = 0.0001$. Ferrum (Iron) also showed a significant decrease in Group 1 (1274.80 ± 230.61 µg/l) relative to Group 2 (2208.00 ± 152.35 µg/l), $p = 0.0001$. **In salivary analysis** Copper levels were

significantly higher in Group 1 ($85.92 \pm 9.10 \mu\text{g/l}$) than in Group 2 ($11.97 \pm 4.16 \mu\text{g/l}$), $p = 0.0001$. Zinc concentrations were also elevated in Group 1 ($220.28 \pm 48.28 \mu\text{g/l}$) compared to Group 2 ($72.09 \pm 17.49 \mu\text{g/l}$), $p = 0.0001$. Ferrum was significantly reduced in Group 1 ($9.09 \pm 3.19 \mu\text{g/l}$) versus Group 2 ($20.30 \pm 4.30 \mu\text{g/l}$), $p = 0.0001$.

CONCLUSION: The study highlights a clear alteration in trace element levels in both serum and saliva among OSMF patients, supporting their potential utility in early diagnosis and disease monitoring.

Keywords: OSMF, (ICP-MS), serum trace element, salivary trace element.

INTRODUCTION-

Oral submucous fibrosis (OSMF) is a progressive, debilitating precancerous condition with an estimated malignant transformation risk of approximately 1%. These pathological alterations contribute to oral mucosal rigidity, leading to trismus and restricted mouth opening. The condition is most commonly linked to habitual practices prevalent in South and Southeast Asia and is increasingly reported in Western nations due to changing lifestyle patterns and migration.⁽¹⁾

Although OSMF can affect individuals across age groups, its highest incidence is reported among young adults aged 25 to 35 years (second to fourth decade). The disease typically follows a slow progression over 2 to 5 years. Since the past few decades, OSMF cases has risen up to 6.42%, results in emergence of a health concern in India. Its high malignant potential, with transformation rates reported as high as 7.6%, contributes significantly to disease-related mortality.⁽²⁾

OSMF has a multifactorial etiology. Identified contributors include areca nut consumption and deficiencies in essential micronutrients such as iron, zinc, and vitamins. Fibrotic changes seen in OSMF are partly linked to compromised collagen and fibronectin integrity. Areca nut alkaloids, particularly arecoline and arecaine, promote fibroblast phagocytosis, while tannins enhance collagen fiber resistance to enzymatic degradation by collagenase.⁽³⁾

Several investigations, including those by Sreelatha S., Yadav, Garg, Shetty, and Fasahat, have demonstrated altered trace element profiles in OSMF—most notably elevated serum and salivary copper levels, along with reduced zinc and ferrum concentrations. Despite these observations, reported findings remain inconsistent, and the existing data lacks clarity.⁽⁴⁾

In light of these inconsistencies and the limited application of inductively coupled plasma mass spectrometry (ICP-MS) in this context, the current study aims to evaluate trace element levels in both serum and saliva using ICP-MS. The objective is to enhance understanding of their role in OSMF pathogenesis and to support early detection and disease monitoring.⁽⁵⁾

MATERIAL AND METHODOLOGY-

- **Design:** Case-control study examining trace element profiles (Cu, Zn, Fe) in patients with oral submucous fibrosis (OSMF) vs. healthy individuals.
- **Setting:** Conducted at TMDC&RC in collaboration with Eko Pro Engineers Pvt. Ltd.; ethics approval obtained and informed consent secured.

Study Population

- **Total participants:** 40 (20 OSMF patients, 20 matched healthy controls).
- **Inclusion criteria:**
 - *Group 1:* Areca nut users meeting Khanna and Andrade's OSMF criteria, no systemic illness or malignancy.
 - *Group 2:* Habit-free individuals undergoing third molar extraction.
- **Exclusion criteria:** OSCC, epithelial dysplasia, systemic illness, prolonged medication use, or refusal to participate.

Equipment & Reagents

- **Key instruments:** ICP-MS (Agilent 7700), microwave digestion system, microtome, Olympus microscope.
- **Reagents:** Suprapur-grade acids, certified standards for Cu, Zn, Fe; formalin, paraffin, hematoxylin-eosin stains.

Sample & Analysis

- **Sample size:** 40 calculated via SPSS ($\alpha = 0.05$, power = 95%, effect size = 1.52).
- **Specimens:** Serum, saliva, tissue biopsies; stored at -40°C .
- **ICP-MS Analysis:** Performed after microwave-assisted acid digestion; concentrations expressed in $\mu\text{g/L}$ with robust calibration and QC protocols.

Clinical staging:

Mouth opening was examined according to criteria established by Khanna and Andrade et al.⁽⁶⁾

Determination: Patient was advised to open mouth at maximum limit. Under good lighting conditions, inter-incisal distance from edges of upper central incisor and lower central incisor was calculated with the help of graduated ruler. This step was repeated three times and average calculated value was noted.

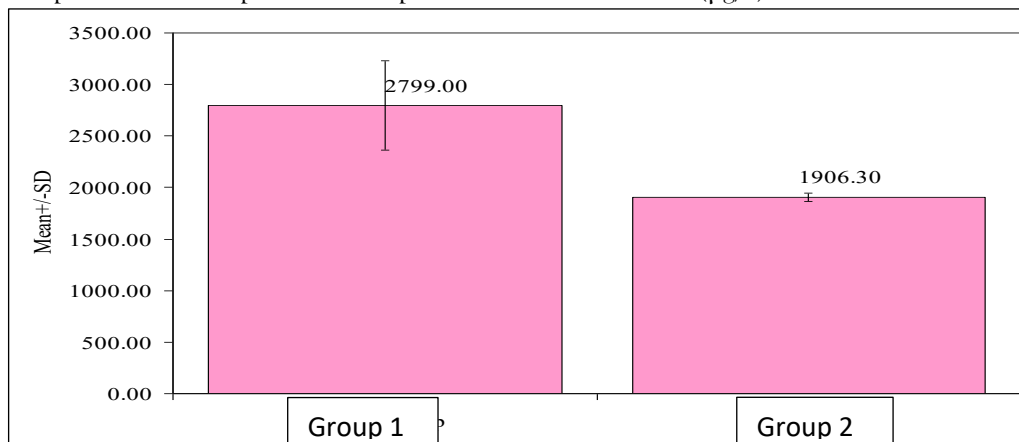
Determination of Histopathological grades:

The histopathological grading of OSMF was done according to Khanna & Andrade et al.⁽⁶⁾

Groups	Epithelial changes	Nature of collagen	Blood vessels	Inflammatory cells	Muscle tissue changes
Group I - very early	Essentially normal and nonkeratinized with occlusal hyperplasia	Fine fibrillar collagen network interspersed with marked edema. A large aggregate of plump young fibroblasts with abundant cytoplasm is evident	Dilated and congested	Consists mainly of polymorphonuclear leucocytes with a few eosinophils	No change
Group II - early	Flattening or shortening of the epithelial rete pegs with varying degrees of keratinization	Juxta-epithelial hyalinization. Collagen present as thickened but separate bundles. Young fibroblasts seen in moderate numbers	Dilated and congested	Chronic inflammatory infiltrate consisting mainly of polymorphonuclear lymphocytes, eosinophils and occasional plasma cells	No change
Group III - moderately advanced	Markedly atrophic, with total loss of rete pegs	Juxtaepithelial hyalinization. Thickened collagen bundles will be faintly discernible, separated by very slight, residual edema. Mature fibrocytes with scanty cytoplasm and spindle-shaped Nuclei	Mostly constricted	Consisted mainly of lymphocytes and plasma cells	Muscle fibres are seen to be interspersed with thickened and dense collagen fibers. In certain areas, the muscle fibers also reveal the beginning of degeneration and irregularity of the sriae
Group IV - advanced	Total loss of epithelial rete pegs Group 4a: without epithelial dysplasia Group 4b: with epithelial dysplasia	Hyalinized as a smooth sheet eliminating all evidence of individual bundles. Fibroblasts were markedly absent within the hyalinized zones, although an occasional thin, elongated cell was seen along the fiber bundles	Obliteration of mucosal blood vessels due to extensive fibrosis	Few chronic inflammatory cells, i.e., lymphocytes and plasma cells	Extensive degeneration of muscle fibers

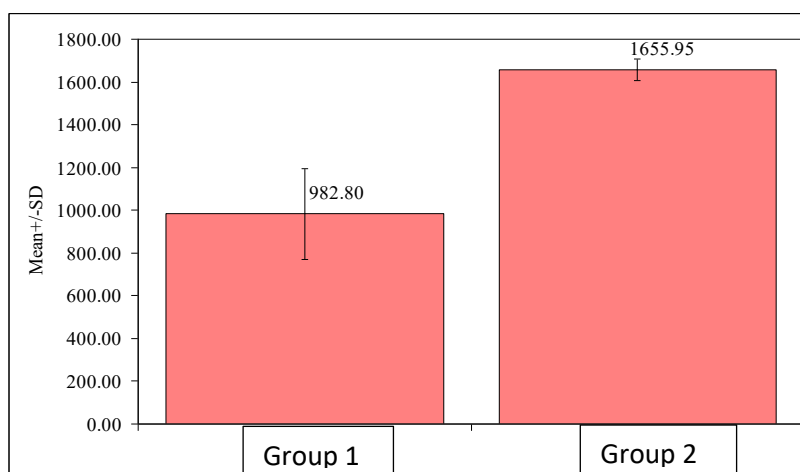
GRAPHS

Graph 1 : Comparison of Group 1 and Group 2 with mean Serum Cu ($\mu\text{g/l}$)



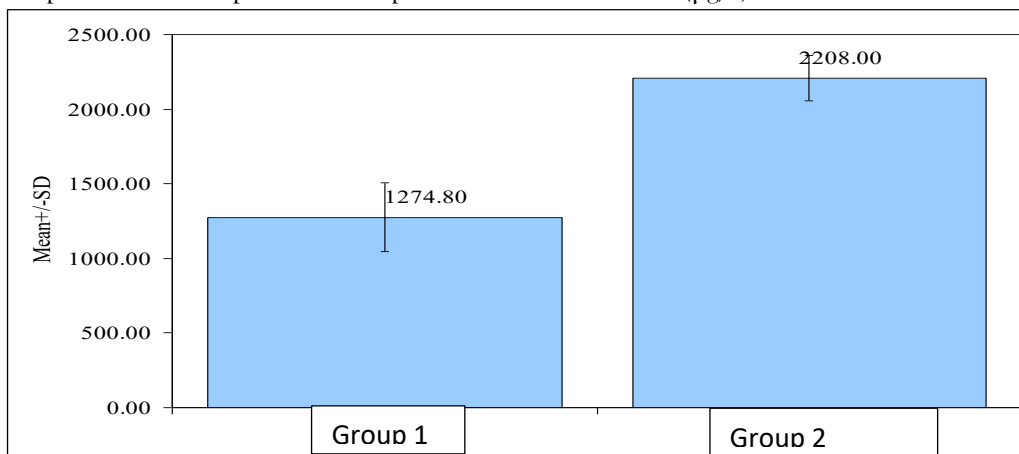
Group 1: OSMF patients, Group 2: Healthy volunteers

Graph 2: Comparison of Group 1 and Group 2 with mean Serum Zn ($\mu\text{g/l}$)



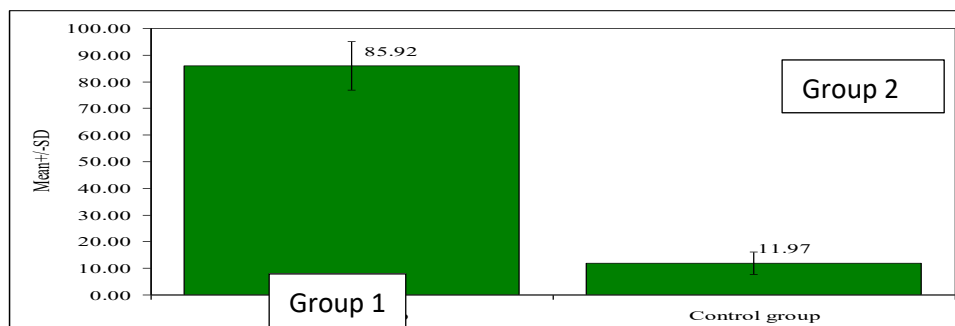
Group 1: OSMF patients, Group 2: Healthy volunteers

Graph 3: Comparison of Group 1 and Group 2 with mean Serum Fe ($\mu\text{g/l}$)

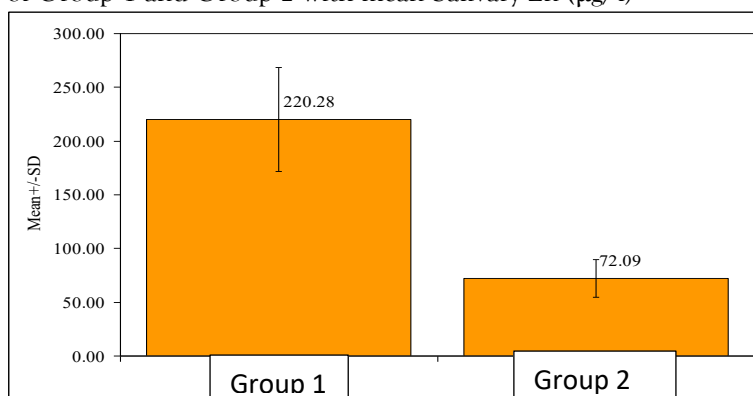


Group 1: OSMF patients, Group 2: Healthy volunteers

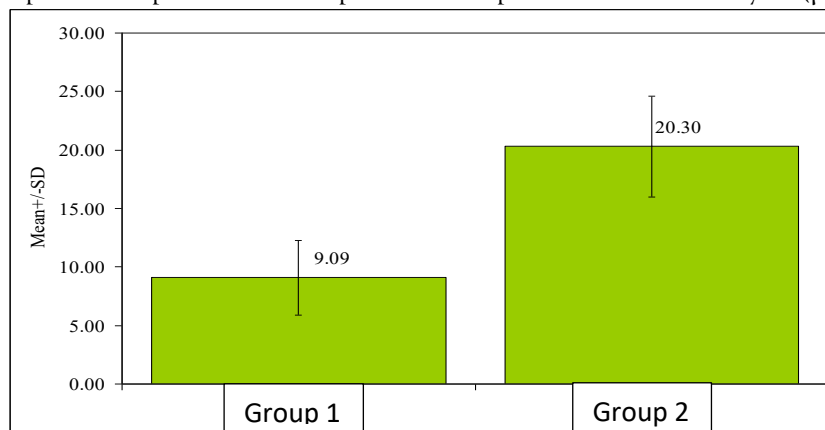
Graph 4: Comparison of Group 1 and Group 2 with mean Salivary Cu ($\mu\text{g/l}$)



Group 1: OSMF patients, Group 2: Healthy volunteers
Graph 5: Comparison of Group 1 and Group 2 with mean Salivary Zn ($\mu\text{g/l}$)



Group 1: OSMF patients, Group 2: Healthy volunteers
Graph 6: Comparison of Group 1 and Group 2 with mean Salivary Fe ($\mu\text{g/l}$)



Group 1: OSMF patients, Group 2: Healthy volunteers



Picture 6: Inductively Coupled Plasma Mass Spectrometer



Picture 7: Mouth opening (Stage 1 OSMF)



Picture 8: Buccal mucosa (Stage 1 OSMF)

RESULTS AND OBSERVATION

A total of 40 participants were recruited and divided into two groups: Group 1 (n = 20), comprising oral submucous fibrosis (OSMF) patients, and Group 2 (n = 20), consisting of healthy volunteers, based on clinical criteria by Khanna and Andrade ^[6].

Demographics and Clinical Findings The mean age in Group 1 was 35.75 years and in Group 2 was 37.15 years, with no statistically significant difference (p = 0.7800). Gender distribution revealed 80% males in Group 1 and 55% in Group 2 (p = 0.0910). Mean mouth opening was significantly reduced in OSMF patients (21.80 ± 8.01 mm) compared to controls (51.15 ± 6.69 mm; p = 0.0001).

Clinical and Histopathological Staging According to the clinical staging system by (Khanna and Andrade ^[6]) 10% of cases were classified as Stage I, 20% as Stage II, 55% as Stage III, and 15% as Stage IV. Histological grading showed: 10% Grade I, 25% Grade II, 45% Grade III, and 20% Grade IV.

Serum and Salivary Trace Element Levels All trace element parameters followed normal distribution (Shapiro-Wilk test). Independent t-tests revealed statistically significant differences between groups (all p = 0.0001):

Parameter	Group 1 (Mean ± SD)	Group 2 (Mean ± SD)	p-value
Serum Copper (µg/L)	2799.00 ± 434.18	1906.30 ± 43.06	0.0001
Serum Zinc (µg/L)	982.80 ± 212.35	1655.95 ± 49.49	0.0001
Serum Iron (µg/L)	1274.80 ± 230.61	2208.00 ± 152.35	0.0001
Salivary Copper (µg/L)	85.92 ± 9.10	11.97 ± 4.16	0.0001

Parameter	Group 1 (Mean ± SD)	Group 2 (Mean ± SD)	p-value
Salivary Zinc (µg/L)	220.28 ± 48.28	72.09 ± 17.49	0.0001
Salivary Iron (µg/L)	9.09 ± 3.19	20.30 ± 4.30	0.0001

Correlation with Clinical Staging Spearman rank correlation showed:

- Strong **positive** correlations: Serum Cu (r = 0.7375), Salivary Cu (r = 0.7286), Salivary Zn (r = 0.8419)
- Strong **negative** correlations: Serum Zn (r = -0.8378), Serum Fe (r = -0.7657), Salivary Fe (r = -0.8146)

Correlation with Histopathological Grading Spearman correlation analysis demonstrated:

Strong **positive** correlations: Serum Cu (r = 0.9359), Salivary Cu (r = 0.9054), Salivary Zn (r = 0.9024)

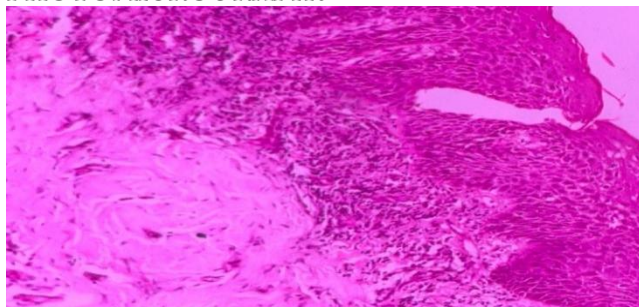
- Strong **negative** correlations: Serum Zn (r = -0.8657), Serum Fe (r = -0.9000), Salivary Fe (r = -0.8856)

Inter-Element Relationships in OSMF Karl Pearson's correlation highlighted:

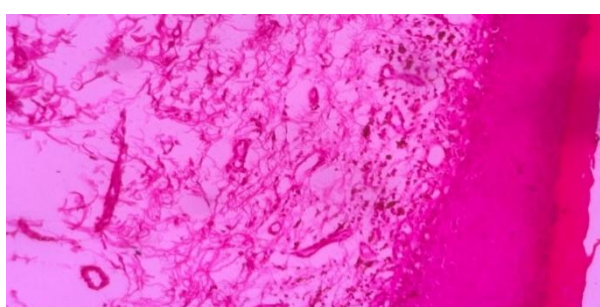
- Strong **positive** relationships: Serum Cu vs. Serum Zn (r = 0.8780), Serum Cu vs. Salivary Cu (r = 0.8049)
- Moderate **positive** correlation: Serum Fe vs. Salivary Fe (r = 0.7640)
- Strong **negative** correlations among various serum and salivary pairs, including: Serum Cu vs. Serum Fe (r = -0.8458), Salivary Fe vs. Salivary Zn (r = -0.9142)

Healthy Controls In Group 2, correlation among serum and salivary trace element levels was generally weak and statistically insignificant (p > 0.05), indicating minimal interdependence.

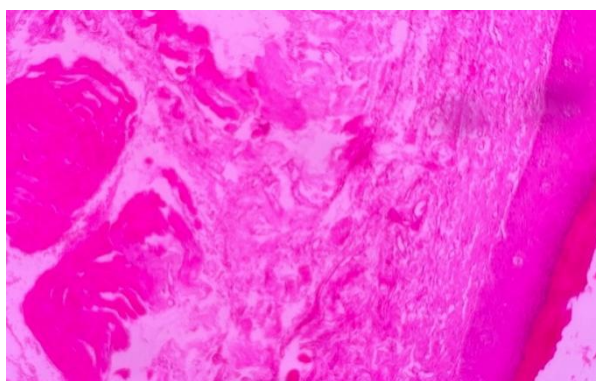
PHOTOMICROGRAPHS



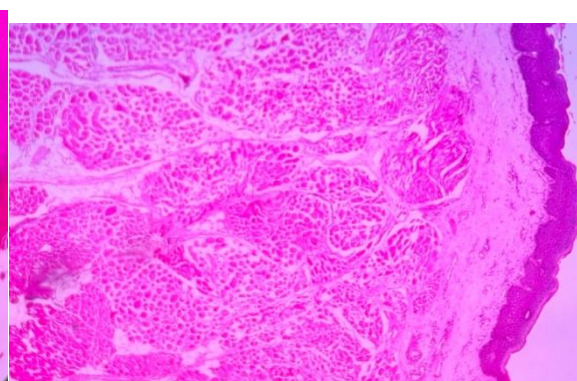
Photomicrograph 1: showing hematoxylin & eosin stained oral mucosa of grade 1 (very early) OSMF at 10x magnification.



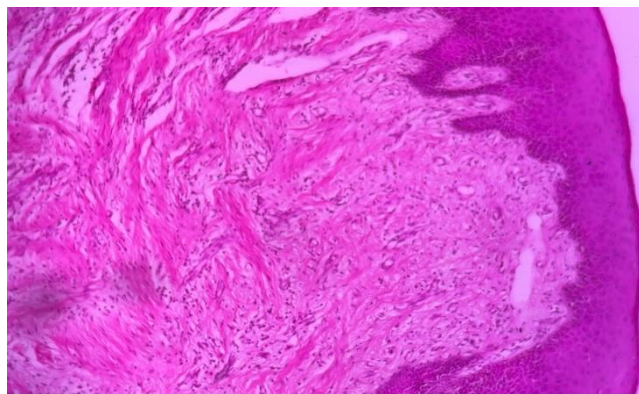
Photomicrograph 2: showing hematoxylin & eosin stained oral mucosa of grade 2 (early) OSMF at 10x magnification.



Photomicrograph 3: showing hematoxylin & eosin stained oral mucosa of grade 3 (moderately advanced) OSMF at 10x magnification.

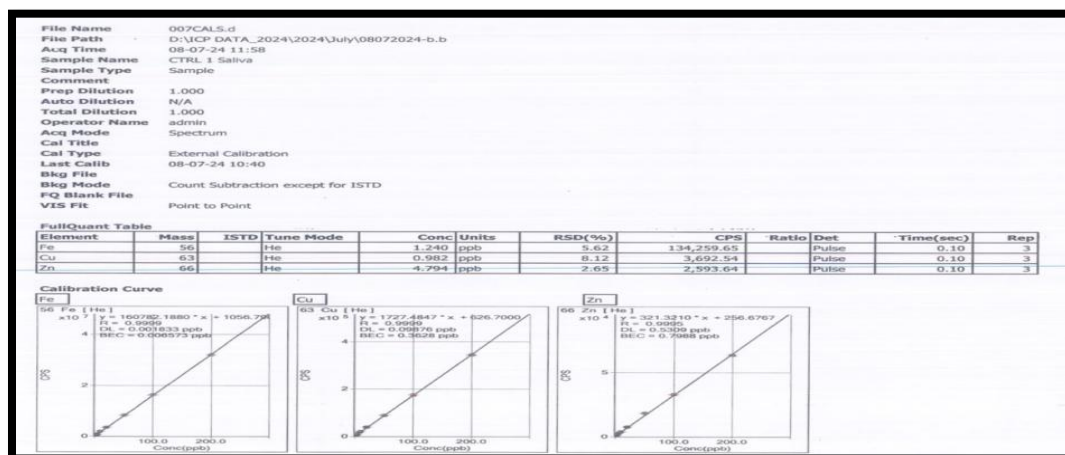


Photomicrograph 4: showing hematoxylin & eosin stained oral mucosa of grade 4 (advanced) OSMF at 10x magnification.

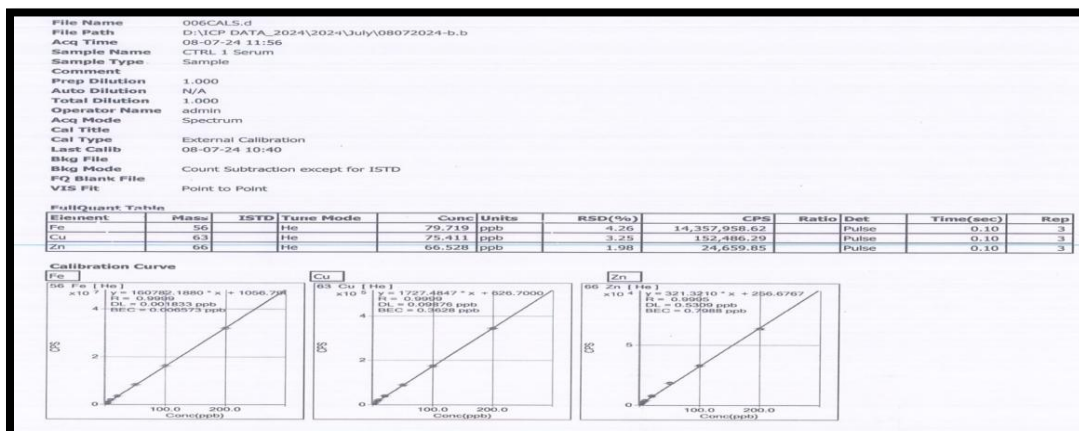


Photomicrograph 5: showing hematoxylin & eosin stained normal oral mucosa at 10x magnification.

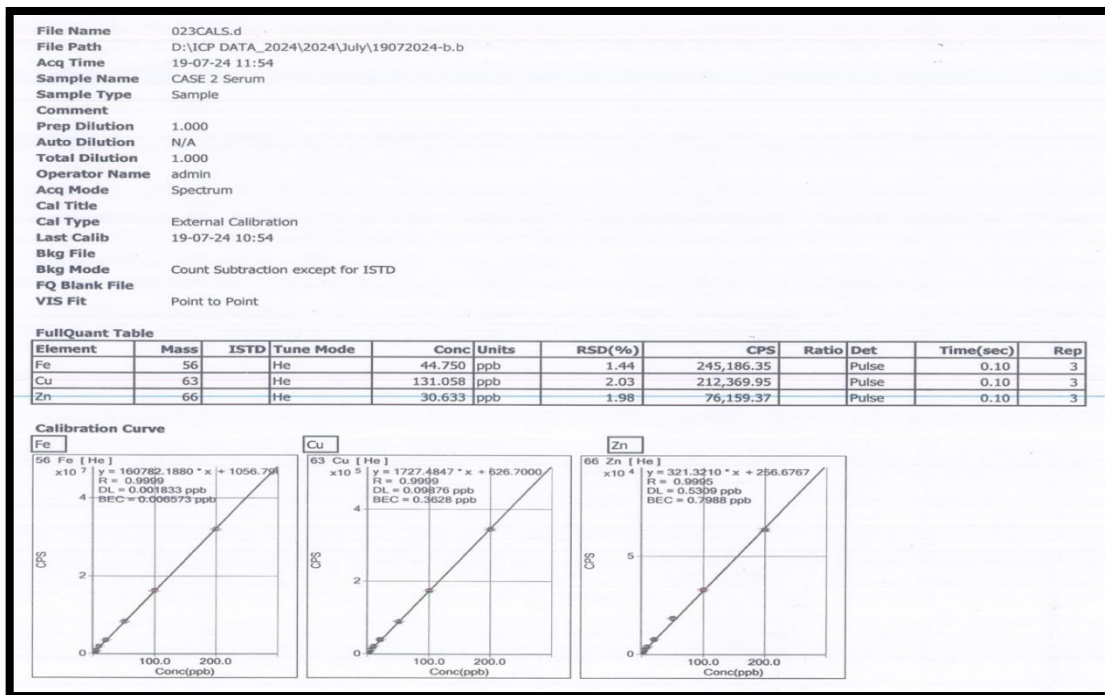
PHOTOGRAPHS



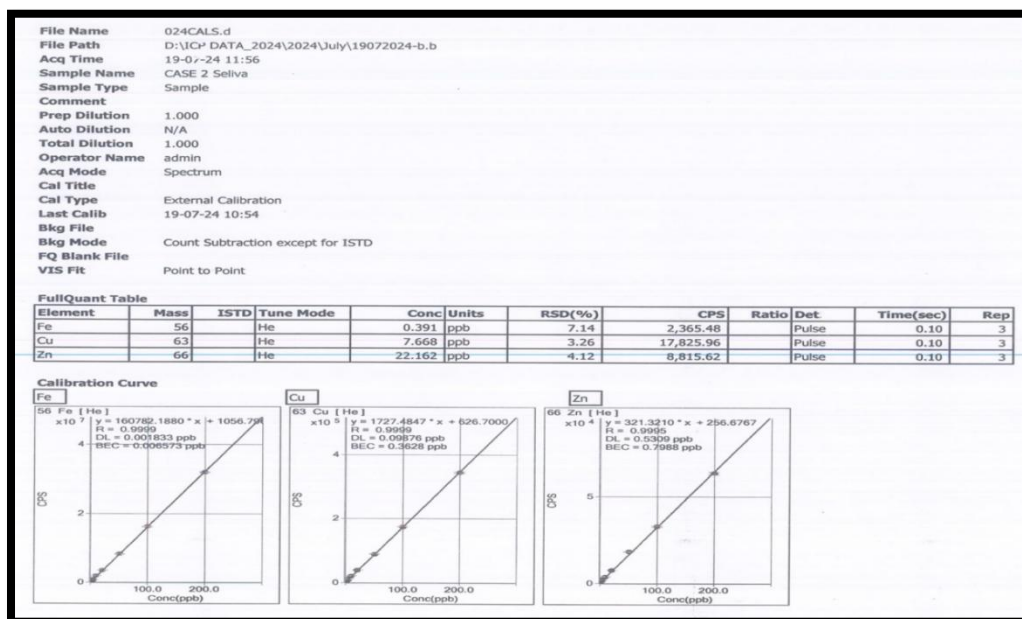
Picture 1: Calibration graphs of Cu, Zn and Fe in serum sample of healthy volunteer



Picture 2: Calibration graphs of Cu, Zn and Fe in saliva sample of healthy volunteer



Picture 5: Calibration graphs of Cu, Zn and Fe in saliva sample of OSMF patient (Grade 4)



Picture3: Calibration graphs of Cu, Zn and Fe in serum sample of OSMF patient (Grade 4)

DISSCUSSION-

Oral submucous fibrosis (OSMF) is a chronic, potentially malignant disorder with multifactorial etiology, predominantly associated with areca nut consumption. The present study investigated serum and salivary levels of copper (Cu), zinc (Zn), and iron (Fe) in OSMF patients and healthy controls, using inductively coupled plasma mass spectrometry (ICP-MS)—a technique known for its high sensitivity and precision, yet underutilized in this domain.

In this study, no statistically significant difference in mean age was observed between OSMF patients and healthy controls ($p = 0.7800$), corroborating the findings of Rajendran et al. and Wahab et al.^[7,8], who noted the condition's prevalence in middle-aged individuals

Biochemical analysis revealed significantly elevated serum copper levels in OSMF patients ($2799 \pm 434.18 \mu\text{g/L}$) compared to controls ($1906.30 \pm 43.06 \mu\text{g/L}$; $p = 0.0001$), in agreement with findings by Garg et al., Wahab et al., Kumar Harshal et al., and Latoo S et al.^[9,8,10,11].

Conversely, serum zinc levels were significantly reduced ($982.80 \pm 212.35 \mu\text{g/L}$ vs. $1655.95 \pm 49.49 \mu\text{g/L}$; $p = 0.0001$), consistent with Rajendran et al., Wahab et al., Hosthor SS et al., and Srilekha et al.^[7,8,12,13].

Serum ferrum levels were also significantly reduced in OSMF patients ($1274.80 \pm 230.61 \mu\text{g/L}$) compared to controls ($2208.00 \pm 152.35 \mu\text{g/L}$; $p = 0.0001$), aligning with Yadav et al., Hande et al., and Ettishree et al.^[14,15,16].

In addition to serum markers, salivary analysis demonstrated significantly elevated copper ($85.92 \pm 9.10 \mu\text{g/L}$) and zinc ($220.28 \pm 48.28 \mu\text{g/L}$) levels, and markedly reduced ferrum levels ($9.09 \pm 3.19 \mu\text{g/L}$) in OSMF patients compared to healthy controls (all $p = 0.0001$). These results support previous studies by Puri et al., Fasahat et al., and Srilekha et al.^[17,18,13], suggesting salivary copper as a non-invasive indicator of lysyl oxidase-mediated fibrosis. This finding diverges from Ayinampudi et al.^[19], who reported a decrease in the copper-to-zinc ratio in premalignant and malignant cases.

Reduced salivary ferrum concentrations observed in this study mirror findings by Bagewadi et al. and Gupta et al.^[20,21], indicating systemic iron depletion and local oxidative stress. Shetty et al.^[22] also reported similar trends, although their results lacked statistical significance, potentially due to differences in analytical sensitivity or sample size.

Clinically, the mean mouth opening in OSMF patients ($21.80 \pm 8.01 \text{ mm}$) was significantly lower than controls ($51.15 \pm 6.69 \text{ mm}$; $p = 0.0001$), confirming trismus as a classical sign of fibrosis, as supported by Gupta et al. and Rao et al.^[20,23]. Notably, 70% of patients in this study were classified in Stages 3 and 4, reinforcing reports by Gupta et al. and Bagde et al.^[20,21], that late-stage diagnosis is often due to persistent habits and delayed clinical intervention.

Histopathologically, 65% of patients were in Grades 3 and 4, a distribution that echoes Santosh et al. and Adhane et al.^[24,25], and underscores the progressive and premalignant nature of OSMF. Correlation with serum copper and iron levels further strengthens their diagnostic and prognostic relevance, consistent with Yesha et al.^[26].

Overall, these results highlight significant trace element imbalances in both serum and saliva of OSMF patients, demonstrating their pathophysiological importance and potential application as non-invasive biomarkers for early diagnosis, staging, and monitoring of disease progression.

CONCLUSION-

This study demonstrated significant alterations in trace element profiles among patients with oral submucous fibrosis (OSMF), including elevated serum and salivary copper levels, decreased serum and salivary iron concentrations, increased salivary zinc, and reduced serum zinc compared to healthy controls. These findings suggest that serum and salivary copper and iron levels—quantified using inductively coupled plasma mass spectrometry (ICP-MS)—may serve as valuable prognostic biomarkers for the identification and monitoring of OSMF.

However, due to the limited sample size and uneven distribution across clinical and histopathological grades, particularly Grades 1 and 4, these results should be interpreted with caution. Further large-scale, multicentric studies are warranted to validate the clinical utility of trace element profiling as a reliable tool for disease surveillance and therapeutic monitoring in OSMF.

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