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ORIGINAL ARTICLE

# Morphometric analysis of oral submucous fibrosis and its correlation with histological staging and clinical severity of trismus

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Morphometric analysis;
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**Abstract** *Aim:* To quantify the histopathological changes in oral submucous fibrosis morphometrically and to correlate those findings with the histological grading and clinical severity of trismus.

*Methods:* A total of hundred histological sections of oral submucous fibrosis were analysed morphometrically by using interactive image analysis system (Image Pro-Plus,V 6.0). Histological staging and the severity of trismus were then compared with the morphometry results. ANOVA and Pearson's chi square tests were applied using the software SPSS V. 13.0 for statistical analysis.

*Results:* The thickness of the epithelium and subepithelial collagen showed no statistically significant differences between the different stages ($p$ value $> 0.05$). However, blood vessel density, mean blood vessel area and mean diameter of the vessels were indirectly proportional to the histological stages ($p$ value $< 0.001$). Histological stages directly correlate the frequency of trismus, but the severity of trismus showed relation neither with the staging nor with the degree of collagenization, measured morphometrically ($p$ value $> 0.05$).

*Conclusions:* The thickness of the epithelium and subepithelial collagen should not be included in the histological staging criteria of oral submucous fibrosis. Probably the degree of hyalinization of

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collagen fibres and involvement of muscle fibres are more important in causing trismus, rather than a simple increase in the subepithelial collagen thickness.



## 1. Introduction

Oral submucous fibrosis (OSF) is an irreversible precancerous condition of the upper aerodigestive tract.[1–3] With progression, patient develops trismus clinically. The alkaloid, arecoline released from the areca nut appears to increase the deposition of collagen, the major extracellular matrix molecule in the subepithelial zone.[4,5] This accumulation increases with the severity of the disease.[6,7]

In the present study the biopsies of lesions from clinically diagnosed [palpable bands on the buccal mucosa] OSF were analysed morphometrically and findings were correlated with the histological staging as well as with the clinical severity of trismus – to rationalize whether those pathological changes have any practical implication in disease progression and also to find out any relation between the clinical severity of OSF and its histological stages. There are limited studies in the literature in which the pathological changes in OSF were quantified morphometrically.[8,9]

## 2. Materials and methods

### 2.1. Ethical consideration

This study has been conducted in full accordance with ethical principles and has been independently reviewed and approved by the ethics board. All the subjects gave written consent. We preserved patient anonymity and did not use any personal information or photograph/s of any patient.

### 2.2. Patients and specimens

The study was conducted in the Department of Oncopathology, Medical College, Kolkata and Department of Pathology, Midnapore Medical College during the period of July, 2010 to June, 2012. On clinical suspicion of having OSF (palpable white bands in the buccal cavity), the patients were biopsied by the concerned surgeon after taking written consent from the representative site particularly from the periphery of the lesion to compare it with the unremarkable areas. Tissues were first sent to the Oncopathology Department where histopathological examination of the specimens is performed. Cases bearing adequate sampling indicated the presence of epithelial layer, subepithelial zone including the muscle layer considered for further evaluation. Histological staging of OSF was done. Before making the diagnosis, the staging criteria of OSF were circulated among the pathologists to minimize subjective error. We used the criteria proposed by Pindborg and Sirsat (1966),[10] who described four consecutive stages based upon sections stained with haematoxylin and eosin.

#### 2.2.1. Very early stage

Characterized by fine collagen dispersed with marked oedema, prominent firbroelastic response dilated and congested blood vessels and inflammatory cells (mainly polymorphs and eosinophils).

#### 2.2.2. Early stage

Early hyalinization in juxta epithelial area with thickened separate bundles of collagen and clumps of young fibroblasts in moderate number.

#### 2.2.3. Moderately advanced stage

In this stage collagen is moderately hyalinized, the amorphous change starting from the juxta-epithelial basement membrane. Occasionally thickened collagen bundles are seen separated by slightly residual oedema. The fibroblast response is less marked. Blood vessels are either normal or constricted as, a result of increased surrounding fibrous tissue. Inflammatory exudates consist of lymphocytes, plasma cells and occasional eosinophils.

#### 2.2.4. Advanced stage

Collagen becomes completely hyalinized and seen as smooth sheets with no separate bundles discernible. Oedema is absent. Hyalinized areas are devoid of fibroblasts, although their elongated cells or vestigial nuclei were seen at rare intervals along the fibre bundles. Blood vessels are completely obliterated or narrowed. Lymphocytes and plasma cells are variably present.

Then the slides were sent to Department of Pathology of Midnapore Medical College for unbiased second opinion as well as morphometric analysis. If we found any discrepancy in diagnosis between these two departments those cases were rejected. Fortunately only three cases were rejected on this ground. Thus a total number of hundred histological sections of OSF were staged and simultaneously analysed morphometrically by using interactive image analysis system (Image Pro-Plus, V 6.0). Paraffin embedded sections of 3–4 μm thickness were stained routinely with Haematoxylin/Eosin, Van Gieson's picric acid and acid fuchsin stain and Masson's Trichrome stains in the Onco-pathology department. The latter two special stains impart different colours to the different connective tissue elements and made the morphometric analysis easy as well as less erroneous by highlighting the area of interest (e.g. endothelial cells, collagen fibres etc.).

### 2.3. Image analysis

Photomicrographs of OSF cases were captured with the help of a camera fitted onto the microscope and directly displayed on the computer monitor. Scanner (4X) was used for measurement of epithelial thickness and collagen thickness, low power objective (10X) for number of endothelial cells, area of blood vessels and the high power objective (40X) for measuring vessel diameter. Before proceeding for morphometry these captured photomicrographs were adjusted according to their magnification with the help of the software. Then the areas of interest were selected and analysis of desired parameters was performed.

A total of five parameters were used for each section – (1) Epithelial thickness, (2) Collagen thickness, (3) Blood vessel density (number of endothelial cells per square micron of the subepithelial zone), (4) Mean blood vessel area, and (5) Average mean luminal diameter of the blood vessels.

### 2.4. Clinical staging of trismus

When patients came to the Department of Oncopathology for collection of their biopsy reports, we followed them up retrograde for the presence or absence of trismus. The interincisional distance was measured in each patient by using a slide calipers scale. This post diagnosis measurement of trismus allowed us to avoid any bias during histological staging. The severity of trismus was staged as follows:

Stage 0 – No trismus; mouth opening $\geqslant$45 mm.
Stage I – Mild to moderate trismus; restricted mouth opening 20–44 mm.
Stage II – Severe trismus; mouth opening $\leqslant$20 mm.

For clinical staging of trismus we followed the criteria proposed by Kirankumar K et al.[11]

Clinicopathological correlation was done with histological staging of OSF and clinical staging of trismus.

### 2.5. Data analysis

Data obtained from the morphometric analysis were finally transported to the excel sheet and the average value of each parameter was calculated. ANOVA test was applied for each parameter to judge the statistical significance of the differences in data between the different stages of OSF. Two-tailed $p$ values $< 0.050$ were considered statistically significant. Finally the clinical staging of trismus was correlated retrograde with the histological staging and their morphometric findings. Pearson's chi square test was applied for that purpose and two-tailed $p$ values $< 0.050$ were considered statistically significant.

## 3. Results

Of the total hundred cases of OSF, histologically 36 were of very early stage, 29 of early stage and 28 of moderately advanced stage and seven cases were advanced stage [Fig. 1]. Morphometrically the variation in epithelial thickness was not consistent with the stages of OSF ($F = 0.81$, $p > 0.05$); however all the cases of advanced stage had atrophied epithelium. Very early stage cases did not show any measurable amount of collagenization. Between the different stages of OSF the collagen thickness showed no statistically significant differences ($F = 0.85$, $p > 0.05$). Number of endothelial cells per square micron [Fig. 2] of the subepithelial zone consistently decreased with the increasing stages of OSF ($F = 14.3$, $p < 0.001$). However, Early stage cases showed no significant differences from the Very early stage, but Moderately advanced stage and Advanced stage showed a highly significant statistical difference from the same. The number of blood vessels is directly proportional to the vessel reaction, which may be either due to an increased number of endothelial cells or blood vessel density. The mean blood vessel area and the mean vessel diameter showed a marked increase in early stage. The luminal diameter in advanced stage showed near obliteration

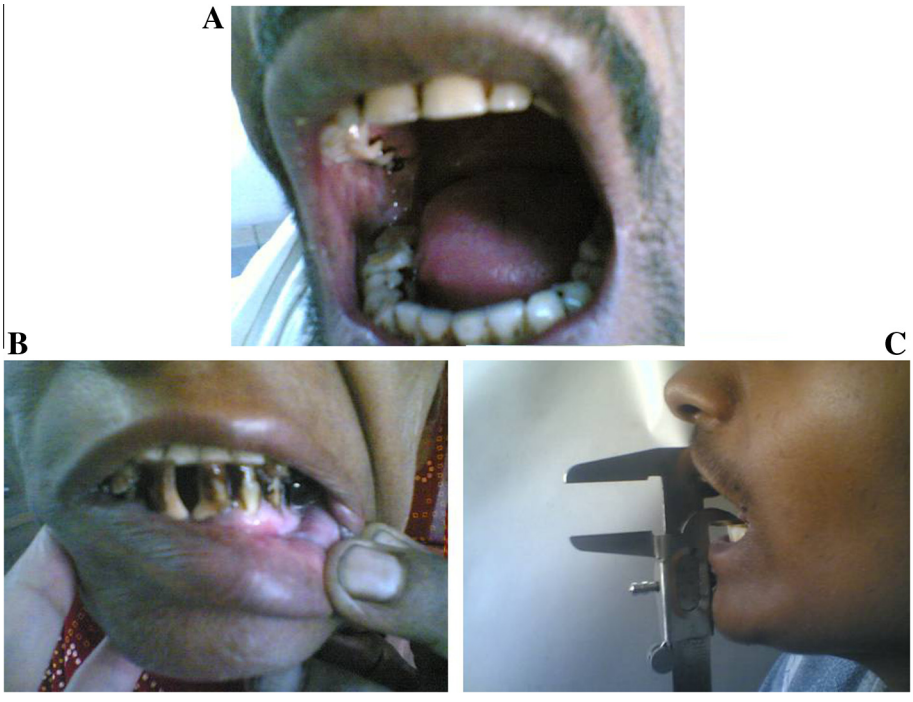


**Figure 1** Patient of OSF with normal mouth opening (No trismus – very early stage disease). White bands are seen on the right buccal mucosa (A), OSF involving the gingivolabial sulcus with tobacco stained teeth (B), and OSF advanced stage, the interincisional distance was measured in each patient by a slide calipers scale (C).

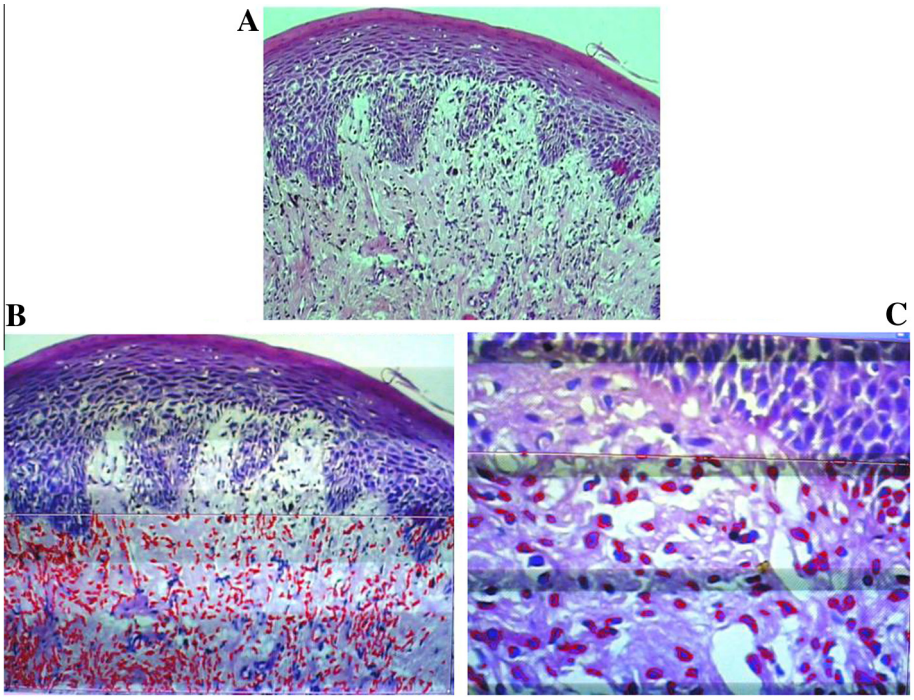


**Figure 2** OSF – very early stage showing hyperkeratinized squamous epithelium, subepithelial numerous dilated blood vessels, inflammatory infiltrates and melanin incontinence indicating an inflammatory process (H&E × 100) (A). Morphometric analysis with the help of the software showing endothelial cells in the subepithelial zone (red colour) and counted (B and C).

**Table 1** Morphometric analysis of different stages of OSF[a] and their comparison.

| Parameters | Very early stage | Early stage | Moderately advanced stage | Advanced stage | ANOVA $p$ value |
|---|---|---|---|---|---|
| Epithelial thickness (μm) | 173.22648 | 169.00886 | 162.50167 | 147.61577 | $F = 0.79$ $p > 0.05$ |
| Subepithelial Collagen thickness (μm)[c] | – | 421.7006 | 441.2798 | 475.8018 | $F = 0.88$ $p > 0.05$[c] |
| No. of endothelial cells/Sq. micron area)[b,d] | 410.0667 | 251.6566 | 211.5667 | 85.6666 | $F = 13.2$ $p < 0.001$ |
| Mean Blood vessel area/Sq. micron area)[b] | 31.5038 | 585.1401 | 164.2813 | 7.7992 | $F = 175.0$ $p < 0.001$ |
| Blood vessel mean luminal diameter (μm) | 5.8996 | 11.8592 | 8.0659 | 0.9818 | $F = 29.3$ $p < 0.001$ |

[a] Oral Submucous Fibrosis.
[b] Low power field.
[c] Compared only between early, moderately advanced and advanced stage.
[d] Proportional to the number of blood vessels.

of the lumen (Table 1). Thus between different stages the mean blood vessel area and the mean vessel diameter showed statistically highly significant differences ($F = 169.0$ and 27.3 respectively, $p < 0.001$).

When trismus was correlated with the histological staging and morphometric findings it was found that the frequency of trismus was directly proportional to the histological stages of OSF – advanced the histological stages, more the number of cases having trismus. However, the severity of trismus showed relation neither with the histological staging nor with the degree of collagenization, measured morphometrically (Tables 1 and 2).

## 4. Discussion

In the literature there are few studies regarding the vascular changes in OSF for comparison, one by Rajendran R et al., 2005 and another one by Fang CY et al., 2000.[8,9] To the best of our knowledge, there are no studies in the literature on image analysis of OSF regarding epithelial thickness and thickness of collagenization, therefore these parameters of the present study could not be compared.

In the present study we found that in OSF the overlying epithelium may have varying thickness regardless of the stages of OSF. However, advanced stage disease is commonly associ-

**Table 2** Comparison between histological staging and clinical severity of trismus.

| Histological grades of OSF[a] | Trismus present [b] No.(%)[c] | Clinical stages of trismus | | |
|---|---|---|---|---|
| | | Stage 0[d] (no trismus) No. (%) | Stage I[e] (mild to moderate trismus) No. (%) | Stage II[f] (severe trismus) No. (%) |
| Very Early stage ($N = 36$) | 05 (13.9) | 31 (86.1) | 02 (40) | 03 (60) |
| Early stage ($N = 29$) | 10 (34.4) | 19 (65.5) | 03 (30) | 07 (70) |
| Moderately advanced stage ($N = 28$) | 16 (57.1) | 12 (42.9) | 09 (56.2) | 07 (43.8) |
| Advanced stage ($N = 07$) | 05 (71.4) | 02 (28.6) | 03 (60) | 02 (40%) |
| Total ($N = 100$) | 36 | 64 | 21 | 15 |
| Chi square $p$ value | 0.001 | 0.381 | 0.930 | 0.280 |

[a] Oral submucous fibrosis.
[b] Presence of trismus irrespective of clinical staging.
[c] Percentage calculated out of total numbers of cases of respective grades.
[d] Stage 0 – Mouth opening $\geqslant$45 mm.
[e] Stage I – Restricted mouth opening 20–44 mm.
[f] Stage II –Mouth opening $\leqslant$20 mm.

ated with atrophy that may be due to chronicity of the disease. Pindborg and Sirsat (1966) also suggested that the overlying epithelium is either atrophic or hyperplastic, often hyperkeratotic.[10]

Again the progression of the disease was not found to be related to the degree of collagenization. This morphometric finding correlates with the observation made by us in the present study as well as by T Rooban et al. and by Kirankumar K et al. in another two different studies that the severity of trismus does not correlate with the stages of OSF.[11,12] Therefore, the statement that trismus occurs due to increase in subepithelial collagen deposition is an oversimplification as was concluded by some authors in some studies.[6,7] Probably hyalinization of the collagen fibres (in which they become stiff) rather than the simple increase in collagen thickness and involvement of muscle fibres (collagen fibres in-between muscle bundles) along with the site of involvement appear to be more important in causing trismus. Pindborg and Sirsat described the very early stages by the presence of fine fibrillar collagen dispersed with marked oedema with increasing thickness in higher stages of disease.[10] Huang IY, Shieh TY also found that accumulation of collagen fibres increases with the severity of the disease.[7]

In the initial stages there is an increase in the number of blood vessels along with dilatation and congestion – early stage showing highest dilatation. That may be due to compensatory or reactive to the environmental insults in the form of alkaloid (from areca nut), chronic irritation or infection etc. At the later advanced stages probably these mechanisms become decompensated due to persistent insults resulting in constriction or obliteration of the blood vessels along with a decrease in their number. These findings are similar to the study results of Fang CY et al. who showed the increase in micro vessel quantity and quantity density in the early stages and the decrease in micro vessel quantity, quantity density, micro vessel area and area density in the middle and the late stages. They concluded that the presence of micro vessel hyperplasia occurred in the early stages of oral submucous fibrosis.[9] Similarly, Pindborg and Sirsat suggested dilatation and congestion of blood vessels in the early stages and constriction to obliteration at the later stages.[10] In the present study we found that maximum dilatation occurs in early stage with a decrease in the subsequent stages. Moderately advanced stages showed dilatation more than Very early stage but less than early stage. Advanced cases showed almost luminal obliteration.

The above findings differ very much rather just an opposite to that of Rajendran R et al. who assessed quantitatively the mucosal vascularity in oral sub mucous fibrosis by image analysis.[8] They found the mean vascular density to be more or less same in the test and control samples ($F = 0.82$, $p > 0.05$) and the mean vascular percentage area and the mean vascular luminal diameter had an increasing trend as the disease progresses ($F = 8.63$, $p < 0.01$ and $F = 34.1$, $p < 0.001$, respectively).

## 5. Conclusion

We concluded that the epithelial thickness and thickness of the collagen should not be included in the histological staging criteria of OSF as they do not seem to have any practical implication on prognosis or occurrence of trismus. Vascular luminal size is inversely related to advancement of OSF stages.

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