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*RESEARCH ARTICLE*

# A HISTOCHEMICAL AND IMMUNOHISTOCHEMICAL STUDY IN ORAL SUBMUCOUS FIBROSIS.

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*Manuscript Info* *Abstract*

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## Introduction:-

Disease follows its own rules, neither those of kings or slaves. In India and Southeast Asia, the use of smokeless tobacco in various forms is very popular. This habit which usually involves chewing of a betel quid (Combined areca nut, betel leaf, tobacco and slaked lime)[1] has lead to the development of a unique generalized fibrosis of the oral tissues called Oral submucous fibrosis.

**Pindborg & Sirsat** in 1966 defined submucous fibrosis as "an insidious, chronic disease affecting any part of oral cavity and sometimes the pharynx. Although occasionally preceded by and / or associated with vesicle formation, it is always associated with a juxta-epithelial inflammatory reaction followed by a fibro-elastic change of the lamina propria, with epithelial atrophy, leading to stiffness of the oral mucosa and causing trismus and inability to eat".[2]
The pathogenesis of OSF is still unclear, but a number of hypotheses have been postulated by Pillai *et al.* [3] The histological appearances of OSMF indicate an excessive deposition of collagen in the lamina propria with or without adequate breakdown, suggesting a loss of the homeostatic mechanisms controlling collagen turnover and consequent fibrosis, functional impairment and irreversible alteration of tissue architecture.[4]

The principal cells implicated as a source of extracellular matrix in areas of fibrosis are fibroblasts. Accumulation of connective tissue matrix is secondary to factors such as cytokines and growth factors. Fibroblast growth factors play an important role in the pathogenesis of oral submucous fibrosis.[5]

Human fibroblast growth factor (FGF) family contains 22 proteins (FGF1 to FGF23, with no FGF number 15) that regulate a plethora of physiological processes in both developing and adult organism. These proteins play crucial roles in normal development, in the maintenance of tissues, and in wound healing & repair & they have also been implicated in a wide range of pathological conditions including tumorigenesis & metastasis**.** They act on cells of meso-, ecto-, endo- dermal origin, and they cause changes in migration, morphology & function as well as proliferation.[6]

In the present study, an attempt is made to evaluate bFGF expression immunohistochemically in fibroblasts, endothelial cells and connective tissue stroma of clinically and histologically proven cases of OSMF and to correlate this with its histological grades. The apparent co-localization of bFGF in areas of collagen deposition is analyzed and the nature of fibers and its distribution in the submucosal tissue are assessed by histochemical stains and picrosirius red stain.

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## Materials And Methods:-

### Tissue material

The material for the study included 30 formalin-fixed paraffin-embedded tissue blocks of histologically proven cases of OSMF between the years 2007 and 2011, which had both the epithelia and the connective tissue. Normal oral mucosal tissue was used as control. 5 um thick sections were cut. A case series analysis was carried out by immunohistochemistry for bFGF expression in fibroblasts, endothelial cells and stroma. Special stains like picrosirius red and Verhoeff's hematoxylin were carried out in all these cases to assess the nature and distribution of connective tissue fibers in different histological grades.

### Immunohistochemical analysis

The immunohistochemical analysis for bFGF was performed using streptavidin–biotin–peroxidase technique. Paraffin-embedded OSMF tissues were cut into 5-um-thick sections and taken onto poly L Lysine microslides. The sections were brought to water after deparaffinization and for antigen retrieval, sections were boiled in sodium citrate buffer (pH 6.0) in a pressure cooker, following which the slides were allowed to cool in citrate buffer and then washed in distilled water. After a brief rinsing in phosphate buffered saline (PBS), sections were treated with 10% goat serum at room temperature for 30 min to block any non-specific antigenic sites. The sections were then incubated overnight at $4^0$C with monoclonal antibody against bFGF (monoclonal antifibroblast growth factor, Biognex Co) with suitable controls. After washing in PBS, sections were incubated with biotinylated secondary antibody at room temperature for 30 min (Biogenex Co.) Following a PBS wash for 5 min thrice, sections were treated with 3,3'-diaminobenzidine tetra-hydrochloride (DAB) (Biogenex Co). The slides were counterstained with Meyer's hematoxylin, subsequent to which sections were dehydrated, cleared and mounted with DPX and cover slipped.

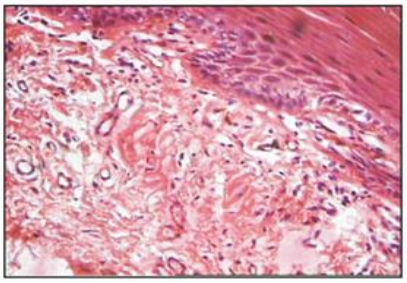
The slides were observed using a light microscope at 40X magnification and reviewed for the expression of bFGF in relation to the fibroblasts, endothelial cells and the fibrous stroma. For the study group, in each case, three fields were randomly selected and the expression of bFGF was evaluated using a 40 X objective. The endothelial staining of normal blood vessel, known to be immunoreactive for bFGF, was used as an internal positive control, and the sections stained without the primary antibody served as negative controls.

The intensity of immunohistochemical reactions were semi quantitatively assessed by two independent observers to remove any possible bias, using a modification of the criteria given by Jankowski and colleagues[7] in 1994 as: + Weakly positive (some staining present but weak), ++ Moderately positive (moderate staining greater than any background staining) and +++ Strongly positive. The bFGF expressivity with respect to these parameters was then compared with the histological grades of OSMF proposed by Sirsat and Pindborg .[2]

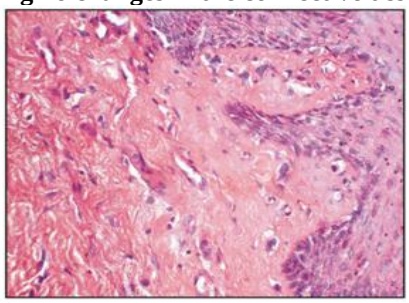
### Histochemical Analysis

The bFGF expressivity was compared with the histological grades of OSMF proposed by Sirsat and Pindborg using Verhoeff's hematoxylin (as coarse or fine fibers). Its distribution adjacent to the vessel, deeper connective tissue and sub-epithelium were studied. Identical sections were evaluated for the amount and distribution of collagen fibers using picrosirius red stain in three different zones (Sub-epithelial zone, intermediate zone, peripheral zone) in various grades of oral submucous fibrosis. Three groups of polarizing colors were made as GY= Greenish yellow, YO= Yellowish orange, RO= Reddish orange**.** Evaluation of the areas, showing transition from greenish-yellow, yellowish-orange to reddish orange birefringence, in same slides under light microscopy was done to see if the colour change was associated with any change in thickness of the collagen fibers.

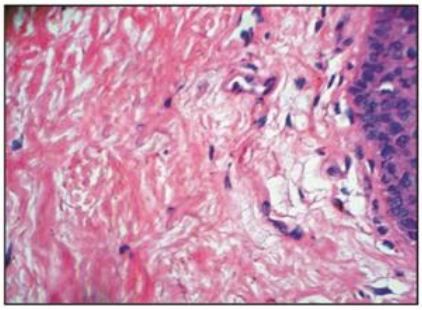
**Haematoxylin & eosin staining (40X)**



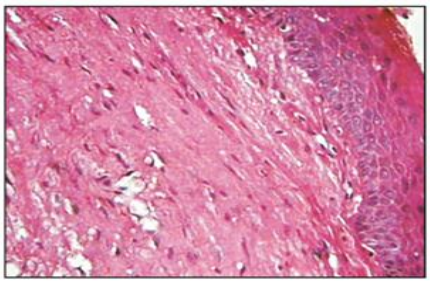
**Photomicrograph of oral mucosa showing no changes in the connective tissue and taken as positive control**



**Photomicrograph of very early/early OSMF showing collagen as separate bundles, many plump fibroblasts and congested blood vessels**

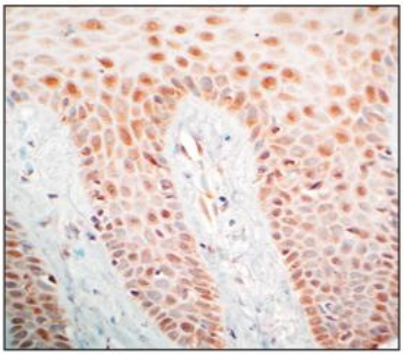


**Photomicrograph of moderately advnced OSMF showing thickened collagen bundles, fewer fibroblasts and normal or constricted blood vessels**

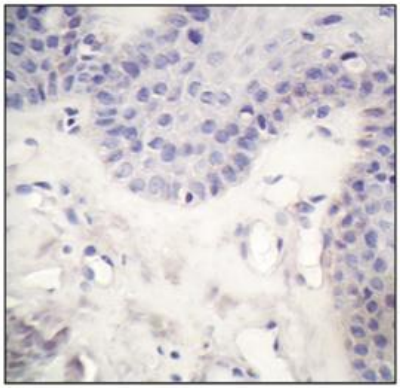


**Photomicrograph of advnced OSMF showing collagen bundles in the form of sheets, absent rete pegs, fewer fibroblasts and obliterated or narrow blood vessels**

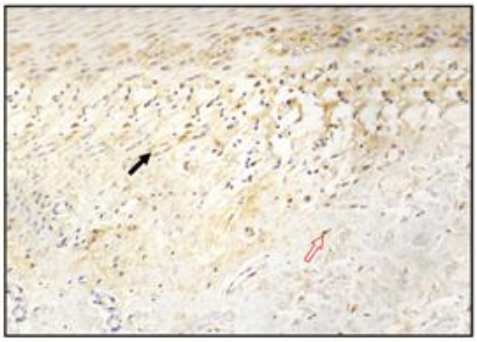
**Immunohistochemical staining by basic fibroblast growth factor (bFGF) 40X**



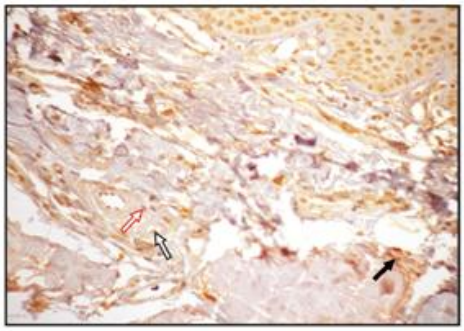
**Photomicrograph of oral mucosa showing bFGF expression in basal/parabasal layer, fibroblasts, and in endothelial cells and taken as positive control**



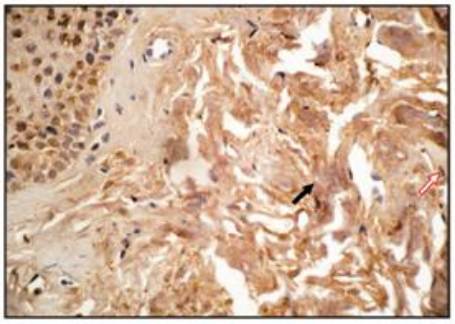
**Photomicrograph of OSMF tissue section showing no immunoreactivity to bFGF in absence of primary antibody and used as negative control**



**Photomicrograph of strongly positive bFGF expression in fibroblasts, in endothelial cells in very early/early OSMF cases**

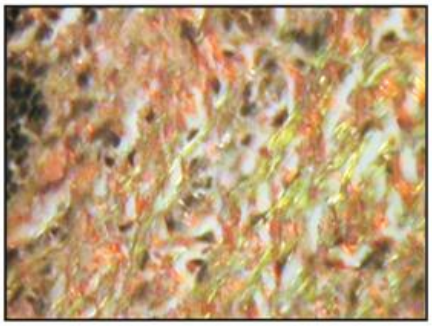


**Photomicrograph of OSMF tissue section showing bFGF expression which is less in fibroblasts and in endothelial cells and more in connective tissue stroma**

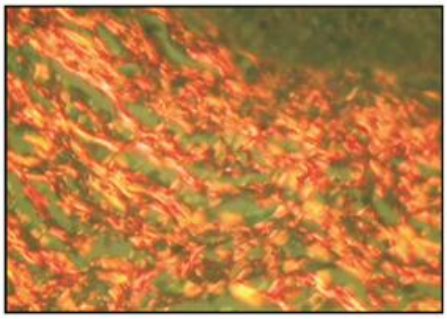


**Photomicrograph of weakly positive bFGF expression in fibroblasts and strongly positive expression in stroma seen in advanced OSMF cases**

**Picrosirius red staining (40X)**

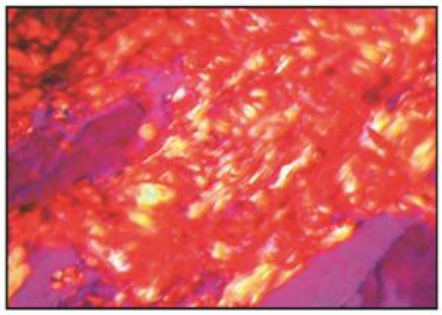


**Photomicrograph showing greenish yellow color in oral mucosa and taken as positive control**



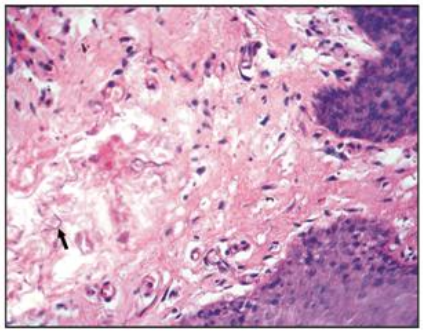
**Photomicrograph showing greenish yellow to yellowish orange color in very early/early OSMF**

**Photomicrograph showing yellowish orange to reddish orange color in moderately advanced OSMF**

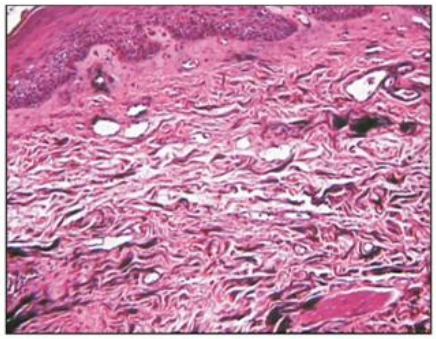


**Photomicrograph showing reddish orange color in advanced OSMF**

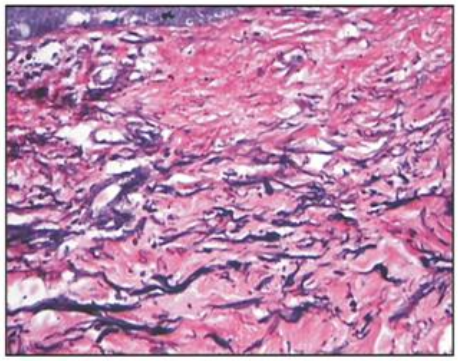
**Verhoeff's haematoxylin staining (40X)**



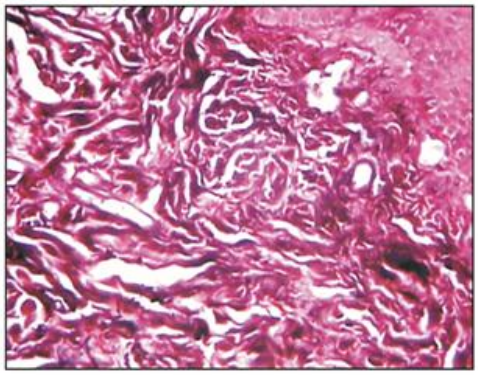
**Photomicrograph of oral mucosa taken as positive control showing fine uniformly distributed fibres in connective tissue**



**Photomicrograph of early OSMF showing coarse fibres distributed in deeper connective tissue**



**Photomicrograph of moderately advanced OSMF showing coarse fibres distributed in deeper connective tissue and a few in superficial lamina propria**



**Photomicrograph of advanced OSMF showing coarse and thick fibres throughout connective tissue stroma and also around blood vessels**

**Statistical Analysis**

**For immunohistochemical staining**

The intensity of immunohistochemical reactions for anti-bFGF was semi-quantitatively assessed by two independent observers to remove any possible bias. To minimize the inter-observer variability, the scores of both observers were subjected to Kendall's tau-b test. Chi-square test was applied to statistically evaluate the intensity of staining in fibroblasts, endothelial cells and stroma. P-values of <0.05 and <0.001 were considered to be significant and very highly significant, respectively. The inter-group relation was studied by performing a chi-squared test to determine if the association had any significance and a p value less than 0.05 was considered to be significant.

**For picrosirius red and verhoeff's haematoxylin staining**

Chi-square test was used for determining the p value i.e probability value. P-values of <0.05 and <0.001 were considered to be significant and very highly significant, respectively. The inter-group relation was studied by performing a chi-squared test to determine if the association had any significance and a p value less than 0.05 was considered to be significant.

## Results and observation:-

The present study was a retrospective study in which samples were selected based on diagnosis after hematoxylin and eosin (H&E) staining. Thirty tissue blocks of OSMF were stained with H & E and graded as very early or early, moderately advanced and advanced OSMF. The normal oral mucosa used as a control tissue was stained for comparison. Out of 30 patients of our study, 22 (73.33%) were males and 8 (26.66%) were females (Table 1).

**Table 1:-**Sex distribution in various grades of OSMF

| Sex | Frequency | Percent |
|---|---|---|
| **Male** | 22 | 73.33% |
| **Female** | 8 | 26.66% |
| **Total** | 30 | 100.0% |

Associated habits were recorded. Gutka chewing was the most commonly elicited habit (Table 2).

**Table 2:-**Comparison of habits in different subjects of OSMF

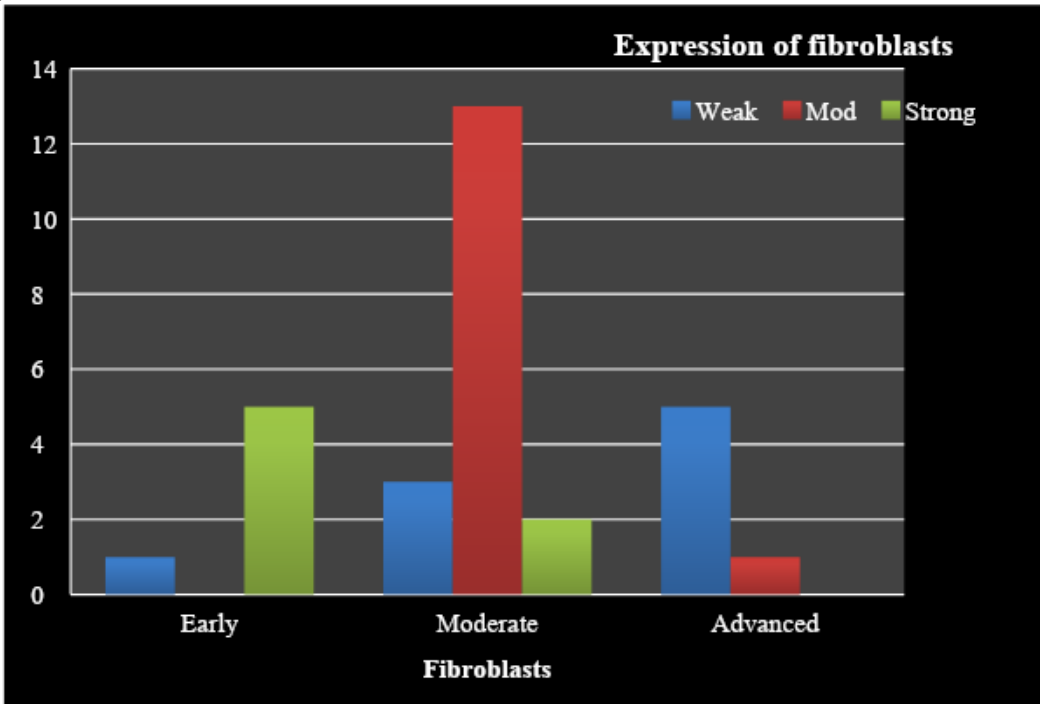
| Habit | Cases | Percentage |
|---|---|---|
| Gutka | 12 | 40% |
| Bidi | 1 | 3.3% |
| Gutka/ tobacco | 3 | 10% |
| Gutka /quid | 1 | 3.3% |
| Betel nut | 2 | 6.6% |
| Gutka and hukka | 1 | 3.3% |
| Bidi/Hukka | 1 | 3.3% |

| | | |
|---|---|---|
| Supari | 1 | 3.3% |
| Gutka and alcohol | 1 | 3.3% |
| Tobacco | 4 | 13.33% |
| Quid | 2 | 6.6% |
| Pan masala | 1 | 3.3% |
| Total | 30 | 100% |

**Evaluation of Immunohistochcemical staining**

**bFGF expression in fibroblasts :**

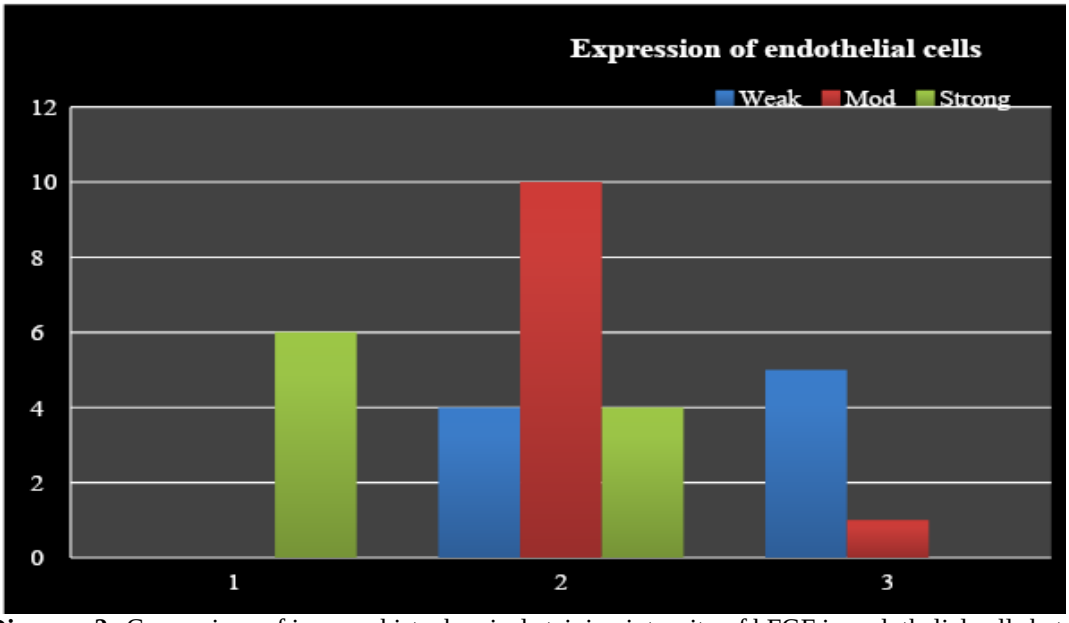
The bFGF expression in the fibroblast was found to be weakly positive (+) in nine cases, moderately positive (++) in 14 cases and strongly positive (+++) in seven cases. On applying Chi-square test for the intensity of staining in relation to fibroblasts, a p value of 0.001 was obtained which was considered to be very highly significant. (Bar diagram 1).




**Bar Diagram 1:-**Comparison of immunohistochemical staining intensity of bFGF in fibroblasts between different grades of OSMF

**bFGF expression in endothelial cells:**

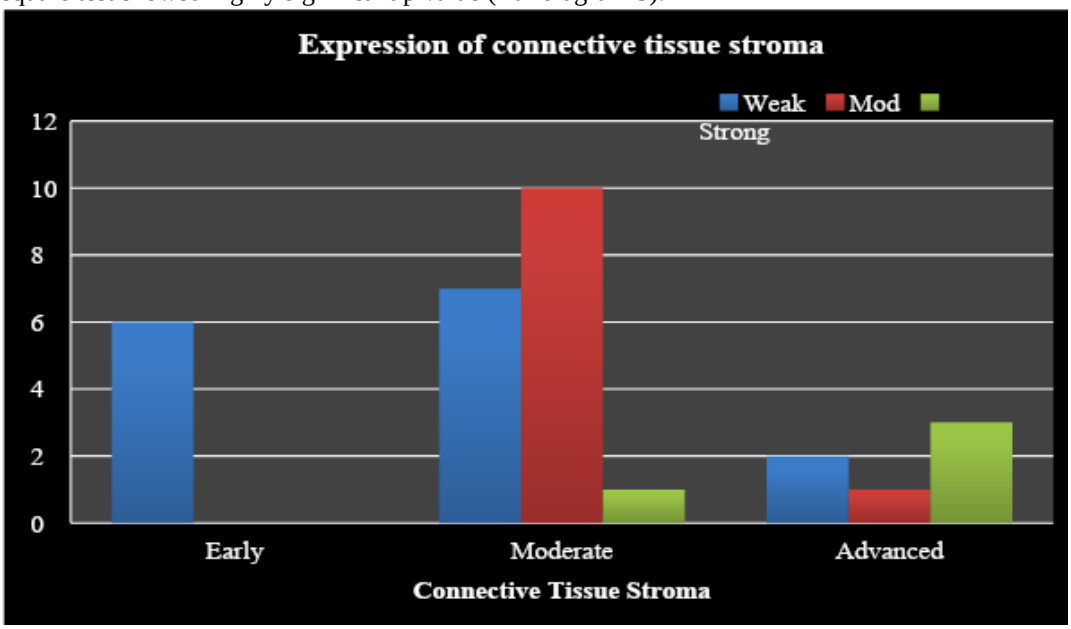
When endothelial cells were evaluated for bFGF expression, nine cases showed weakly positive (+) staining, 11 cases showed moderately positive (++) staining and 10 cases showed strongly positive (+++) staining. Overall analysis was found to be significant (Bar diagram 2).




**Bar Diagram 2:-**Comparison of immunohistochemical staining intensity of bFGF in endothelial cells between different grades of OSMF

**bFGF expression in fibrous stroma**

When expression in fibrous connective tissue was studied, weakly positive (+) staining was seen in 15 cases, 11 cases showed moderately positive (++) staining and four cases of OSMF showed strongly positive (+++) staining. Chi-square test showed highly significant p value (Bar diagram 3).




**Bar Diagram 3:-**Comparison of immunohistochemical staining intensity of bFGF in connective tissue stroma between different grades of OSMF

**p value analysis:**

**In fibroblasts**

The statistical significance of inter group relation was also studied by performing a Chi square test. p value less than 0.04 was considered to be significant. The comparison of early OSMF with moderate OSMF showed a p value of 0.002 which was considered to be highly significant; early OSMF with advanced OSMF gave a p value of 0.013,

which was significant and the comparison of moderate OSMF with advanced OSMF gave a p value of 0.01, which was again significant.

**In endothelial cells**:
Comparison of early OSF with moderate OSF gave a p value of 0.05 which was not significant and same was observed with the comparison of early OSF with advanced OSF, which gave a p value of 0.2 and with comparison of moderate OSF with advanced OSF in which a p value of 0.1 was observed.

**In connective tissue stroma**
Comparison of early OSMF with moderate OSMF showed a p value of 0.034 which was significant; and the same was true when compared in between moderate and advanced cases which gave a p value of 0.032. Comparison of early OSF with advanced OSF gave a p value of 0.05 which was considered as non significant.

From these observations, a decrease in the expression of bFGF intensity was noted in fibroblasts and endothelial cell as we higher up from early to moderately advanced to advanced OSF cases. In contrast, the intensity of bFGF expressivity in the connective tissue stroma increased from early to advanced cases of OSMF.

**Evaluation of picrosirius red stain**
Picrosirius staining was studied in three different zones of OSMF: Sub-epithelial zone, Intermediate zone and Peripheral zone. Buccal mucosal tissue was taken as control which showed greenish yellow color in subepithelial, intermediate and peripheral zone. As OSMF ranges from very early/early to moderately advanced and advanced stages, the color of the tissue changes from greenish yellow to yellowish orange to reddish orange.

**Subepithelial zone:**
When expression in subepithelial zone of OSMF was studied, out of six cases of early OSMF, interestingly, all six cases showed yellowish orange color while out of 18 cases of moderately advanced OSMF, 13 cases showed reddish orange color and rest five showed yellowish orange color. In advanced OSMF cases which were six in total, 4 showed reddish orange color and rest 2 yellowish orange color. Statistical analysis was done and it showed a p value of 0.007 which was highly significant. (Table 3)

**Intermediate zone:**
Yellowish orange birefringence was recorded more in early OSMF cases (all 6 cases) while reddish orange birefringence was more in moderately advanced OSMF (15 cases) and out of 6 cases of advanced OSMF, 4 showed reddish orange color and 2 yellowish orange. Statistical analysis showed p value of 0.006 which was highly significant. (Table 3)

**Peripheral zone:**
When expression in peripheral zone of OSMF was studied, Out of 6 cases of early OSMF, interestingly, 5 cases showed yellowish orange color and 1 reddish orange color while out of 18 cases of moderately advanced OSMF, 11 cases showed reddish orange color and 7 cases showed yellowish orange color. All 6 cases of advanced OSMF showed yellowish orange color (Table 3)

**Table 3:-**Distribution of colour birefringence in OSMF and oral mucosa

| **Grades** | **Sub epithelial zone** | | | **Intermediate Zone** | | | **Peripheral Zone** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **GY** | **YO** | **RO** | **GY** | **YO** | **RO** | **GY** | **YO** | **RO** |
| **Early** | 0<br>0% | 6<br>100% | 0<br>0% | 0<br>0% | 6<br>100% | 0<br>0% | 0<br>0% | 5<br>83.33% | 1<br>16.66% |
| **Moderate** | 0<br>0% | 5<br>27.77% | 13<br>72.22% | 0<br>0% | 3<br>16.66% | 15<br>83.33% | 0<br>0% | 7<br>38.88% | 11<br>61.11% |
| **Advanced** | 0<br>0% | 2<br>33.33% | 4<br>66.66% | 0<br>0% | 2<br>33.33% | 4<br>66.66% | 0<br>0% | 6<br>100% | 0<br>0% |
| **Oral Mucosa** | 1<br>100% | 0<br>0% | 0<br>0% | 1<br>100% | 0<br>0% | 0<br>0% | 1<br>100% | 0<br>0% | 0<br>0% |
| **Statistics** | Chi Square Value - 11.035<br>p Value-0.0115 sig | | | Chi Square Value-14.842<br>p Value-0.002 hs | | | Chi Square Value- 9.457<br>p Value-0.0238 sig | | |

| **GY- Greenish Yellow** | **YO- Yellowish Orange** | **RO- Reddish Orange** |
|---|---|---|

We observed that as the stages advanced, the polarization color of thick fibers significantly showed orange red-red color and there was a considerable decrease in the greenish yellow color. This is due to the fact that the overall number of thin fibers diminished markedly as the stages of disease progressed. In advanced stages, as the collagen fibers appeared hyalinized and tightly packed, delineation into thick and thin fibers was not observed. However, the tight packing of collagen fibers resulted in orange red- red color throughout the sections.

**Evaluation of Verhoeff's hematoxylin stain:**
As the changes taking place in fibrous connective tissue stroma was evident with respect to each of the progressive grades of OSMF, Verhoeff's hematoxylin stain was done to assess the nature of fibres in OSMF tissues in different grades.

On staining the tissues with Verhoeff's hematoxylin stain, the fibres were seen as uniformly distributed black colored fine fibres in the lamina propria of normal mucosa. There was an evident change in the nature of fibres in OSMF cases as there was an increase in the coarse fibres in the deeper tissues in the early OSMF graded by H and E. However, coarse fibres tended to become thicker in the moderately advanced OSMF and the distribution of these fibres extended above to involve the superfical lamina propria. The thickened coarse fibres in the advanced OSMF was rather diffuse and were seen in both the superficial and deeper lamina propria as well as around the blood vessels. (Table 4)

**Table 4:-**Comparison of staining intensity of Verhoeff's hematoxylin in different zones of various grades of OSMF

| **Grades** | **Sub epithelial zone** | | | | **Intermediate Zone** | | | | **Peripheral Zone** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | - | + | ++ | +++ | - | + | ++ | +++ | - | + | ++ | +++ |
| **Early** | 6<br>66.66% | 0<br>100% | 0<br>0% | 0<br>0% | 1<br>100% | 4<br>100% | 1<br>5% | 0<br>0% | 0<br>0% | 2<br>33.33% | 4<br>22.22% | 0<br>0% |
| **Moderate** | 3<br>33.33% | 8<br>88.88% | 7<br>63.63% | 0<br>0% | 0<br>0% | 0<br>0% | 17<br>85% | 1<br>80% | 0<br>0% | 4<br>66.66% | 13<br>72.22% | 1<br>50% |
| **Advanced** | 0<br>0% | 1<br>11.11% | 4<br>36.36% | 1<br>100% | 0<br>0% | 0<br>0% | 2<br>10% | 4<br>20% | 0<br>0% | 0<br>0% | 1<br>5.55% | 5<br>50% |
| **Statistics** | Chi Square Value- 23.77<br>P Value-0.0006, vhs | | | | Chi Square Value- 36.67<br>P Value-0.0001, vhs | | | | Chi Square Value- 19.26<br>P Value-0.004, hs | | | |

## Discussion:-

OSMF is a chronic oral condition first described by Joshi[8] in 1953. Clinically, it is characterized by blanching of the oral mucosa associated with stiffness of the mucosa. Vesicle formation occurs occasionally. Patients with severe cases have distinct difficulties in chewing, swallowing, and speaking. Many exhibit pronounced atrophy of the lingual papillae.[9]

The high incidence of OSMF in the Indian subcontinent is causally associated with the commonly prevailing habit of chewing areca nut and tobacco. Though the exact mechanism is not known it is believed that areca nut (betel nut) component of betal quid, especially an alkaloid called arecoline, plays a major role in the pathogenesis of OSMF by causing an abnormal increase in the collagen production.[10] Similarly, the flavanoid components of areca nut have been found to have some direct influence on collagen metabolism. It has been found that alkaloid exposure of buccal mucosal fibroblasts results in the accumulation of collagen.[11] A decreased degradation of collagen due to increased cross-linking of the fibres and reduced collagenase activity are found in OSMF mucosa compared to normal oral mucosa. This evidence implies that OSMF may be considered a collagen-metabolic disorder resulting from exposure to areca nuts.[12]

The abnormal accumulation of collagen and other proteins of extracellular matrix (ECM) may be due to the induction of altered cytokine milieu that is critical to the kinetic and synthetic stimulation of activated fibroblasts causing fibrosis.[4]

Numerous cytokines (monokines and lymphokines) are associated with signaling for growth and maintenance of mesenchymal cells including interleukin-1 *(IL-1)*, tumor necrosis factors (TNF-〈 and TNF-®), interferon-gamma (IFN-©), the colony-stimulating factors, and the so-called growth and differentiation factors including transforming growth factors 〈 and ® (TGF-〈 and TGF-®), insulin like growth factor-I (IGF-I), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF). [13] These cytokines have been demonstrated to be produced at the sites of active fibrosis where they appear to be expressed by activated inflammatory cells and other non inflammatory cells including mesenchymal cells, such as fibroblasts and epithelial cells as well.

Of these cytokines, bFGF is important in terms of the direct stimulation of extracellular matrix expression which typifies fibrosis. However, there is enough scientific evidence which indicates that there is involvement of more cytokines in the pathogenesis of fibrosis than.

The bFGF which often interacts synergistically with other growth factors may enhance ECM deposition. Having a high affinity for heparin and similar molecules, their binding causes a conformational change that protects it from proteolysis and denaturation. It acts mainly through a paracrine⁄autocrine mechanism involving high-affinity transmembrane receptors and heparin sulfate proteoglycan low-affinity receptors.[6]

In the present study, we used bFGF for the immunohistochemical study to investigate the possible role of bFGF in the disease progression of OSMF and special stains like picrosirius red and Verhoeff's hematoxylin were also used to study the morphological changes in the connective tissue which allowed clear differentiation between fibrotic areas from adjacent apparently normal connective tissue.

All the cases in our study, which showed strongly positive immunoreactivity in fibroblasts were apparently cases which were graded as early OSMF in routine staining. Our observation of increased cellular expression of bFGF in fibroblasts was in accordance with observations carried out by Haque et al.[4] They studied immunolocalization of cytokines and growth factors and found increased expressivity of bFGF in early OSMF cases with fibroblast influx which further emphasizes the fact that bFGF as a cytoplasmic polypeptide growth regulator, induces the fibroblast activation and possibly acts as an immunomodulatory factor during the early stages of inflammation.[4]

These observations are perhaps the same implied by Knerer et al[14] who indicated that oral fibroblasts are capable of producing a number of proinflammatory cytokines and this stimulation of fibroblasts as well as connective tissue matrix accumulation were secondary to certain stimulatory factors. The habit of chewing gutka was the most probable stimulatory factor in our subjects as majority of them had this habit.

Our observations are well supported by the study done by Kundendu et al[15] in 2008. They studied bFGF expression in OSMF and found that bFGF immunoreactivity was increased in fibroblasts in early OSMF cases. Increased bFGF expression in early stages of the disease was explainable to an initial injury phase because of areca consumption.

The reduced expression of the cellular fibrogenic growth factor in the established disease was perhaps due to the bFGF being released out from differentiated and senescent cells into the ECM which under normal circumstances are intracellular. However, the bioavailability of the FGFs would be locally regulated at the target cell, thus ensuring that their effects are not systemic.[6]

As a potent angiogenic factor and endothelial cell mitogen[6], expression of bFGF in endothelial cells was strongly positive in early cases, while it was reduced in moderately advanced and advanced OSMF cases. This observation was similar to the one elicited by Xu et al[16] who investigated the role of endothelin-1 (ET-1), a potent vasoconstrictor and mitogenic peptide in fibrosis and collagen production. Their findings supported that cell-specific expression of ET-1 may play a role very early in the pathogenesis of OSMF. In addition, this increased bFGF expression in early stages of OSMF was related to microvessel hyperplasia which occurs in the early stages.[17]

Mast cells which are found in the early stages of OSMF are indicated to be a primary source of heparin and may serve as a significant source for heparin binding growth factor, the bFGF, in disease processes.[18]

Pepper et al[19] studied the synergism between VEGF and bFGF and found that VEGF is half as potent as bFGF in inducing the angiogenesis in early stages of OSF which may be the reason of intense staining in early stages of OSF in endothelial cells.

The study done by Haque et al[4] also supported the fact that endothelial cells reacts intensely with bFGF. Most extracellular FGF is sequestrated by binding to heparin sulfate, either on the cell surface or in the extracellular matrix in the presence of TGF β. The resulting complex is resistant to degradative enzymes. The expression of bFGF which showed intense staining in OSF correlates with the present understanding of the excessive deposition of collagen subsequent to cytokine and growth factor synergism.[4]

Our finding of increased expression of bFGF in stroma with increased fibrosis was consistent with that of Morita et al[20] in 1994, who established that heparan sulfate shows enrichment of bFGF-binding domains in fibrotic lesions; and these regions may play an important role in the fibrogenesis through their interaction with endogenous bFGF. Haque et al[4] found that cytokines and growth factors produced by inflammatory cells within the OSMF may promote fibrosis by inducing proliferation of fibroblasts, upregulating collagen synthesis and downregulating collagenase production. The increase in pro-inflammatory cytokines and growth factors may play an important role in the pathogenesis of OSMF.
Thomas et al[21] further confirmed that the principal cells implicated as the source of the ECM in areas of progressive fibrosis are fibroblasts with the phenotypic appearance of myofibroblasts. The myofibroblastic phenotype is known to bring about structural and functional changes in heparan sulfate proteoglycan expression as compared with normal fibroblasts and is associated with impaired responses to FGF-2. The altered stromal distribution of bFGF in OSMF could be because of lower stromal cell concentration and aberrant extracellular deposition of cytokine.

Histochemical analysis of the stroma particularly collagen fibrers can help to assess nature of disease. Picrosirus red stain and polarization microscopy can be employed to study the different types of collagen fibres.[22]

The enhanced birefringence produced by the sirius red stain under polarizing light help in differentiating the different types of collagen fibres such as pro-collagen, intermediate collagen, mature collagen and pathologic collagen fibres. Colour profile of the collagen fibres related to physical aggregation and bio-chemical distribution of fibres. Type -1 collagen corresponds to thick strongly birefringent yellow orange to orange fibres while type -III are weakly birefringent greenish fibres (thin fibres).[23]

The interpretations of the colour profiles exhibited by the collagen fibres given by Junqueira et al. (1982)[24] was considered as standards to interpret the tissue sections in the present study, which ranged from green to greenish-yellow and reddish orange**.** In one of the nuclear magnetic resonance (NMR) studies by Sharf Y et al (1997)[25] showed that colour profile is related to the physical aggregation of collagen fibres. Green to greenish-yellow corresponds to poorly packed collagen fibres, whereas orange red originates from well-packed fibres. Usually, polarization colours of thin normal collagen fibres are green to greenish-yellow, whereas thick fibres range from yellowish-orange through orange to red.

In our study, early OSMF cases showed yellowish orange to orange color while it was predominantly reddish orange in moderately advanced and advanced OSMF cases.

Our study is well supported by study done by Ceena at al[26] who analyzed collagen distribution in different stages of OSMF using the picrosirius red stain under polarizing microscopy for thickness and polarizing colors. Examination revealed, there was a gradual decrease in the green–greenish yellow color of the fibres and a shift to orange red–red color with increase in severity of the disease. Thereby, it appeared that the tight packing of collagen fibres in OSMF progressively increased as the disease progressed from early to advanced stages.

Verhoeff's hematoxylin characterization of the network of elastic fibres in different stages further provided the evidence that the process of fibrosis starts in the deeper sub-epithelial connective tissue stroma. Verhoeff's hematoxylin primarily reacted with coarse (elastic) fibres in the deeper tissues in the early stages and the distribution was in both the deeper and superficial lamna propria in moderately advanced stages and in addition around the blood vessels in advanced cases.

Although these findings could be compared with those revealed by immunohistochemical analysis and ultrastructural studies on collagen in submucous fibrosis[27], related studies are deemed necessary to determine the dynamics of extracellular protein deposition in OSMF. From these observations, it could be inferred that bFGF has a role to play in the mesenchymal transformation secondary to fibroblastic activation and altered vascularity, thus potentiating the disease progression in OSMF. This observed difference in stromal cell expression in different stages of OSMF may complement the morphological distinction of stromal reaction observed with special stains. This feature is especially relevant because the presence or absence of associated fibrosis did not completely influence the pattern of stromal bFGF expression.

We propose from the preliminary observations performed in our analysis that the pathophysiology of OSMF could be described as overlapping, yet sequential events of biochemical alteration occurring at the cellular level, where there is initially a cellular activation and injury phase. During this phase, the endothelium facilitates the migration of mononuclear cells into the interstitium and activated fibroblast begins to populate the interstitium. The stimulus for this efflux could be the chemotactic cytokines playing an important role. Our findings of increased bFGF expression in both the endothelial cells and the fibroblasts could possibly be explained understanding these fundamentals of cell kinetics. Concomitant to the increased matrix protein synthesis, an impaired matrix turnover during this phase is also evident. The latter is due to the increased production of protease inhibitors such as tissue inhibitors of metalloproteinase (TIMP) and plasminogen activator inhibitors (PAI) which inactivate the proteases that regulate matrix turnover. TIMP-1 has cellular effects that are independent of its ability to inhibit MMPs.[28]An emerging theme in the progressive fibrotic disease is the induction of PAI-1, produced by epithelial cells, fibroblasts and myofibroblasts role in OSMF.

The ultimate sequelae to matrix formation are tissue destruction, epithelial atrophy and capillary obliteration. In the final analysis, it is evident that the process of fibrosis is a complicated one with several cellular and molecular mediators interacting in concert. Recently, the direct effect of bFGF-1 and TGF-b on fibroblast proliferation and collagen synthesis using cultured oral fibroblasts have shown opposing effects on growth, differentiation and extracellular matrix accumulation. While bFGF was autorepressive and catabolic, TGF-b has shown to be autoinductive and anabolic, thus representing a part of feedback mechanism controlling stromal growth. However, when bFGF and TGF-b were associated the anabolic effects prevailed.[29]

Contrary to this effect, bFGF was found to be the most potent growth factor in increasing proliferation, glycosaminoglycans synthesis and promoting collagen synthesis in TMJ disk cells.[28] Additional studies to test the effect of bFGF and TGF-b alone and in combination on cultured fibroblasts from OSMF tissues may prove beneficial, as these studies may provide a greater insight into its pathogenesis and offer novel options for therapeutic intervention.

## Conclusion:-

The "balance" of positively and negatively regulating cytokines and the potential for interaction with exogenous factors like areca is considered important in pathogenesis of OSMF. It seems unlikely that any single molecule acting in isolation will be able to trigger the full spectrum of events that typify fibrosis. Picrosirius red stain in conjunction with polarizing microscopy may serve as a specific and sensitive tool in characterizing the nature of collagen fibres in various grades of OSMF.30,31 Additional studies to test the effect of bFGF on fibroblasts from OSMF tissues may prove beneficial, as these studies may provide a greater insight into its pathogenesis and offer novel options for therapeutic intervention. Picrosirius red and Verhoeff's hematoxylin stain can be used as a tool in diagnosis of OSMF.

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