**Pathogenesis of Oral Submucous Fibrosis: The Past and Current Concepts**

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**Abstract**

Oral submucous fibrosis is considered to be a potentially malignant disease caused primarily due to areca nut chewing. Various theories have been put forth since many decades by many researchers about the pathogenesis of oral submucous fibrosis. Still many controversies do exist and modern concepts have been evolved in the current decades. This review attempts to give a bird’s eye view about the various pathogenesis that have been proposed in the past and present.

**Key Words:** Mouth Diseases; Oral Submucous Fibrosis; Pathogenesis; Etiology; Precancerous Condition; Lime-piper Betel Quid; Multifactorial Causality; Betel Nut.

**Introduction**

Oral submucous fibrosis (OSMF) is a chronic disease and a well-recognized potentially malignant condition of the oral cavity characterized by inflammation and a progressive fibrosis of the lamina propria and deeper connective tissues. It is a condition predominantly seen among people of Indian origin, and an epidemiologic survey done a decade ago showed no less than 250,000 cases in India, a figure that must have increased sharply.\(^1,2\) The pathogenesis of OSMF is believed to be multifactorial. Factors that trigger the disease include consumption of chewing areca nut, chilies, nutritional deficiencies, and immunologic processes. The most important risk factor however is the chewing of betel quid and this had been supported by epidemiological, case control, animal experimental and tissue culture studies as well.

A multifactorial model for the pathogenesis of OSMF such as iron and nutritional deficiencies, chronic candidiasis, tobacco, lime, betel quid, genetic abnormalities, Herpes simplex virus (HSV), Human papilloma virus (HPV), autoimmunity etc. have been postulated and are known to have either have direct effect in causing OSMF or an indirect effect by mediating the immune system which is compromised in OSMF.\(^1\) (Flow chart 1)

Pathogenesis is believed to involve juxta-epithelial inflammatory reaction and fibrosis in the oral mucosa, probably due to an increased cross linking of collagen through up-regulation of lysyl oxidase activity. Fibrosis, or the building up of collagen, results from the effects of areca nut, which increases collagen production (e.g., stimulated by arecoline, an alkaloid) and decreases collagen degradation. Thus, OSMF is now considered a collagen metabolic disorder.\(^3\)

Areca nut chewing is known to cause local trauma and injury to the oral mucosa due to its abrasive nature. This could be more severe in users of pan masala and gutkha due to their fine particulate nature, with the high probability of particle adhesion to the traumatized mucosa, leading to morphological changes and membrane damage. This continuous local irritation by pan masala, gutkha or areca nut can lead to injury related chronic inflammation, oxidative stress and cytokine production. Oxidative stress and subsequent Reactive oxygen species (ROS) generation can induce cell proliferation, cell senescence or apoptosis, depending upon the level of ROS production. During chronic exposure, these events can lead to preneoplastic lesions in the oral cavity and subsequently to malignancy.\(^3\)

The role of the constituents of areca nut in the pathogenesis of OSMF has been studied in detail over last two decades. It is apparent that fibrosis and hyalinization of sub epithelial tissues account for most of the clinical features encountered in this condition.\(^4\) Moreover, substantial amount of research on elucidating the etiology and
pathogenesis appear to have been focused on changes in the extracellular matrix (ECM). It was logical to hypothesize that the increased collagen synthesis or reduced collagen degradation as possible mechanisms in the development of the disease. There are numerous biological pathways involved in the above processes and, it was likely that the normal regulatory mechanisms are either down regulated or up regulated at different stages of the disease.

When the betel quid is placed in the buccal vestibule for about 15 minutes to an hour with a frequency of 5 to 6 times a day, it leads to continuous contact between the quid and oral mucosa resulting in absorption and metabolism of the alkaloids in the quid. Further such reactions result in the chronic inflammation causing activation of macrophages and T cells and an increase in the level of cytokines such as IL6, TNF, IFγ and TGF-β. Further, microtrauma produced by the friction of coarse fibers of areca nut also facilitates diffusion of the alkaloids into the sub epithelial connective tissue resulting in juxta epithelial inflammatory cell infiltration.

**Collagen production pathway**

There are three main events in this pathway that is activation of procollagen, elevation of procollagen proteinase levels, and upregulation of lysyl oxidase (LOX activity). The activation of procollagen genes by TGF-β is causing an increased expression of procollagen genes and hence increases collagen level in OSMF. Elevation of procollagen proteinases cleaves C-terminal which plays an essential role in pathogenesis of OSMF.

**Up-regulation of LOX**

The enzyme lysyl oxidase is found to be upregulated in OSMF. This was a copper dependent enzyme and plays a key role in collagen synthesis and its cross linkage. The fibroblasts in OSMF have not only increased lysyl oxidase activities but also potentiate specific growth characteristics. This was evident with the reported cell doubling time of 3.2 days for OSMF and 3.6 days for normal fibroblasts.

**Collagen degradation pathway**

There are two main events that are modulated by TGFβ

- Activation of tissue inhibitor of matrix metalloproteinase gene (TIMPs).
- Activation of plasminogen activator inhibitor gene (PAI).

**Upregulation of cyclo-oxygenase (COX-2)**

Treatment of the buccal mucosal fibroblasts with 80 μg/ml arecoline in culture revealed that COX-2 expression was up-regulated as early as half an hour, indicating this to be an early cellular response to arecoline at transcriptional level. COX-2 expression started to decrease when the arecoline concentration was increased up to 160 μg/ml, and this may be due to cytotoxicity.

**Role of Heat shock proteins (HSP)**

HSP47, is a 47 kDa collagen-binding heat shock protein (HSP), which belongs to the serine protease inhibitor (serpin) superfamily containing a serpin signature sequence. HSP47 mRNA was up regulated by arecoline in human BMFs. Thus, the accumulation of collagen in oral mucosal connective tissue may be caused by a simultaneous effect on HSP47 by areca quid chewing.

**Role of basic fibroblastic growth factor (bFGF)**

The bFGF may either directly stimulate endothelial cell proliferation or facilitate Vascular endothelial growth factor (VEGF) endothelial cell interaction through the modulation of endothelial cell integrin or VEGF receptor expression. Endothelial cell derived IL-1 and bFGF modulate fibroblast properties independently, which supports the hypothesis that altered endothelial cell–fibroblast communication may be involved in the pathogenesis of OSMF.

Spontaneous and stimulated production of cytokines by peripheral blood mononuclear cells (PBMC) from OSMF patients revealed that significant differences in the stimulated versus non-stimulated levels of IL-1β, IL-6, IL-8 and TNF-α but not of INF-γ production. NF-kappaβ expression induced by safrrole in fibroblasts may be mediated by an extracellular signal-regulated protein kinase (ERK) activation and COX-2 signal transduction pathway.

**Role of saliva**

Saliva doesn't play a direct role but in the causing OSMF but it can act as vehicle or an indirect role. The different mechanisms are discussed. Saliva of patients with OSMF contains a thrombin-like substance, identified as fibrin production factor. In contrast, fibrinolytic substances have been
shown in normal saliva. Fibrin production factor in saliva interacts with plasma or fibrinogen in the submucous area of the oral cavity and produces dense fibrosis. Some antithrombin agent or antiproteinn substances could help in stopping the progression of the disease.

Copper content in various betel quid ingredients has been reported to range from 3 to 108 mg/g in areca nut and from 8 to 53 mg/g in pan masala. The iron levels measured were 75 ng/g in areca nut, 132 ng/g in betel leaf, 5.2 ng/g in catechu and 22 ± 256 ng/g in slaked lime samples. In vitro, not only was areca nut induced ROS production enhanced by Fe2+, Fe3+ and Cu2, but formation of 8-oxo-dG in calf thymus DNA was also increased in the presence of Fe2+ and Fe3+. Significant superoxide anion production, assayed by cytochrome c reduction and lipid peroxidation by formation of thiobarbituric acid-reactive substances, was demonstrated in normal human oral keratinocytes following exposure to commercially available gutkha and pan masala extracts. It was noted that there was a significant increase in serum and salivary IgG, IgA levels and decrease in total serum proteins.

Role of Mast cell
Mast Cell Density (MCD) and Micro Vascular Density (MVD) in different grades of OSMF were assessed immunohistochemically and the results showed that when MCD increases there is an exponential increase in MVD proving that lesion is characterized by progressive fibrosis in early stages and there is a failure of degradation or remodeling in the advanced stages. The vesicle formation and symptoms of itching sensation to histamine released from the mast cells suggested the concept of mast cell histamine chain. Mast cell mediators like prostaglandins and leukotrienes are potent secretogouges for the serous and mucous cells and they attribute to the increased salivation seen in OSMF. Histamine could probably attribute to sub mucosal edema seen in early stages of oral submucous fibrosis. Due to increased vasopermeability, eosinophilic chemotactic factor (ECF) is released from the mast cells. This could probably attribute to the eosinophils that are sometimes a part of inflammatory cell infiltrate seen in the early stages of oral submucous fibrosis. Interleukin-5 causes increased proliferation and differentiation of eosinophils. Interleukin-1 from the mast cells could cause increased fibroblastic response and mast cell derived tryptase causes increased production of type-I collagen and fibronectin thereby attributing to the increased fibrosis.

Role of Micronucleated cells and micronuclei
In the patients with OSMF exfoliated cells were obtained by scraping from right and left buccal mucosa and were screened for micronucleated cells and micronuclei. Frequency of micronucleated cells and micronuclei in OSMF patients were high. The variations in the micronucleated cells may be attributed to the factors like ingredients in the quid, frequency of quid per day and differing lifestyle, gender, age and food habits.

Role of lipids
A significant decrease in plasma total cholesterol, HDLC, and triglycerides was observed in OSMF patients. Low levels of cholesterol in the proliferating tissues and in blood compartments could be due to the rapidly dividing cells in malignancies. Several prospective and retrospective studies have shown an inverse trend between lower serum cholesterol and head and neck cancer. A significant decrease in HDL and Apo-A1 levels may be an useful indicator reflecting initial changes occurring in pre-cancerous and neoplastic conditions and it might be a consequence of disease that is mediated by utilization of cholesterol by membrane biogenesis. Precancerous lesions are able to remodel/metabolize lipids for their growth and to generate phospholipids membrane.

Role of Hypoxia
With the progression of the disease process of OSMF, the production of collagen type 1 is increased, and the degradation of collagen is reduced by up to 75%. These changes have been mainly attributed to the alteration of collagen-related genes by chemicals present in areca nut, namely arecoline, arecaidine, guvacine, and guvacoline. Extensive fibrosis of the connective tissue causes reduction of vascularity, resulting in subsequent hypoxia in both fibroblasts and surface epithelia. Hypoxia causes atrophy and ulceration of the epithelium by inducing apoptosis. In addition, the Overexpression of hypoxia-induced factor-1a is seen in OSMF, which indicates changes in cell proliferation, maturation, and metabolic adaptation.
increasing the possibility of malignant transformation. The genetic mechanism of carcinogenesis in OSMF is still to be explained, and with the improvement of knowledge in these areas, better management strategies will be developed.

Role of minerals
Significantly lower levels of hemoglobin and serum iron have been reported in OSMF by many authors. In iron deficiency state, levels of cytochrome oxidase are low, consequently leading to epithelial atrophy. An atrophic epithelium makes the oral mucosa vulnerable to the soluble irritants. Further, lack of iron in tissues causes improper vascular channel formation resulting in decreased vascularity. This leads to derangement in the inflammatory reparative response of the lamina propria resulting in defective healing and scarification. Thus, the cumulative effect of these initiating and promoting factors leads to further fibrosis, which is a hallmark of OSMF.

Levels of circulating immune complexes (CIC), trace elements (copper, iron and selenium) in serum revealed increased circulating immune complex levels in the precancer and cancer patients. Serum copper levels showed gradual increase from precancer to cancer patients. However, serum iron levels were decreased significantly in the cancer group. Selenium levels showed marked decrease in the cancer group. Best predictors for the occurrence of lesions were age, serum iron, CIC, serum selenium in the decreasing order.

Serum zinc levels are decreased in patients with OSMF which can act as indicator for malignant transformation. Copper can up regulate the expression of enzyme lysyl oxidase leading to fibrosis. Copper can stabilize enzyme activity by increasing its half-life. N terminus of exon-1 of lysyl oxidase molecule has copper binding and site this interaction may upregulate the expression of these enzymes. This event leading to crossing of collagen and elastin making it less degradable.

Inductively coupled plasma-mass spectroscopy in oral mucosa of OSMF individuals portray interesting alterations in elemental profile have been noted, indicating a homeostatic imbalance.

Role of Genetic and immunologic factors
Gene-gene interaction analysis revealed that XRCC3 Thr 241 Met had the largest univariate effect followed by XRCC3 Thr 241 Met - NAT2 A857G in men that presents a highly synergistic interaction as one of the potential combinations of single nucleotide polymorphisms (SNPs) to increase the risk of OSMF in men if exposed to arecanut or smokeless tobacco usage. Connective tissue growth factor (CTGF/CCN2) is associated with many human fibrotic disorders and was found to overexpress in OSMF. Microtrauma could lead to the release of thrombin. Thrombin produced by microtrauma may contribute to the pathogenesis of OSMF by up-regulating CCN2 expression. This effect could be mediated by protease-activated receptor-1, reactive oxygen species, apoptosis signal-regulating kinase 1, and c-Jun NH(2)-terminal kinase pathways and prevented by epigallocatechin-3-gallate.

Molecular epidemiological studies have provided that several allelic variants of polymorphic glutathione s-transferases (GSTs) show impaired enzyme activity and are suspected to increase the host's susceptibility to various cancers. The frequency of both the GSTM1 and GSTT1 null genotypes was higher in the OSMF cases. Pathogenesis of OSMF may be epithelial-driven and involve arecoline-dependent up-regulation of αvβ6 integrin.

The MMP1 single-nucleotide polymorphism (SNP) was genotyped and frequency of 1G/2G or 2G/2G promoter genotypes having the 2G allele was associated with higher enzymatic activity and significantly increased in OSMF. Arecanut chewing and alcohol abuse may enhance the expression of the 2G allele of MMP1 genes in OSMF cases. Association of adenosine insertion/deletion polymorphism (-1171 5A-6A) in the MMP-3 promoter region and its expression of MMP-3 genotype associated with the 5A alleles, may have an important role in the susceptibility of the patients to develop OSMF. FAS A(-1377)-G(-670) vs. G(-1377)-A(-670) haplotype was correlated with the malignant potential of OSMF. Heme oxygenase-1 expression was significantly higher in OSMF specimens and expressed mainly by fibroblasts, endothelial cells, and inflammatory cells. Arecoline was also found to elevate HO-1 mRNA and
protein expression in a dose-dependent manner.\textsuperscript{43}

Microarray analysis to characterize the mRNA changes of 14,500 genes to identify novel biomarkers of OSMF was performed in which the expression of Loricrin and Cartilage oligomeric matrix protein (COMP) showed statistically significant association with histologic grade of OSMF. COMP was found to be overexpressed frequently in patients with the habit of areca nut chewing for more than 4 years. CYP 3A5 was revealed an inverse correlation with histologic grade.\textsuperscript{44} High-resolution mapping technique has revealed that a small number of discrete hot-spot LOH loci appeared in 47-53% of the OSMF tissues studied. Many of these LOH loci were previously identified regions of genomic instability associated with carcinogenesis of the HNSCC.\textsuperscript{45} Inducible nitric oxide synthase (iNOS) modulates angiogenesis in human models and this information could be extrapolated in elucidating the pathophysiology of OSMF. Significant vasodilation noticed in these cases argues against the concept of ischemic atrophy of the epithelium. Vascularity and iNOS expression helped to explain the vasodilatation noticed (sinusoids) in this disease; NO being a net vasodilator.\textsuperscript{46} Antinuclear (ANA), antismooth muscle (SMA), antigastric parietal cell (GPCA), antithyroid microsomal (TMA), and antireticulin antibodies were demonstrated in OSMF patients.\textsuperscript{47} Endothelin-1 (ET-1), a potent vasoconstrictor and mitogenic peptide, in fibrosis and collagen production was expressed in OSMF patients.\textsuperscript{48} Effect of arecoline on vimentin, an intermediate filament, and its expression in human buccal mucosal fibroblasts on exposure to various levels of arecoline revealed dose-dependent cytotoxicity for cultured fibroblasts. Immunohistochemical assay also revealed that vimentin expression was much higher for OSMF specimens.\textsuperscript{48}

Patients with OSMF had a higher frequency of the G allele at position +49 on exon 1 of Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) compared with controls. CTLA-4 polymorphism had also been associated with certain autoimmune diseases such as SLE, IDDM, Graves’ disease, Hashimoto thyroiditis, multiple sclerosis and rheumatoid arthritis.\textsuperscript{49} The expression of Transglutaminase 2 (TGM-2) was studied in OSMF tissues and significant overexpression was observed. Arecoline induced TGM-2 mRNA and protein expression as well as TGM-2 activity were increased in human gingival fibroblast cells. The addition of methocholine hemihydrate (M-2 muscarinic acetylcholine receptor selective antagonist) or 8′-bromo-cAMP abolished arecoline-mediated TGM-2 induction, suggesting a role for M-2 muscarinic acid receptor and a repressor role for cAMP.\textsuperscript{50}

Biopsy specimens from OSMF cases were subjected for staining by immunohistochemistry for p53 protein. Few cases being positive for p53 protein show mild degree of dysplasia. Arecanut chewing and/or smoking in OSMF and OSCC cases may play a role in the p53 over expression.\textsuperscript{51} OSMF microarray revealed differential expression of 5288 genes. Among these, 2884 are upregulated and 2404 are down regulated. Immunohistochemistry confirmed upregulation of transforming growth factor-beta 1 (TGF-beta beta 1), TGFBIp, THBS1, SPP1, and TIG1 and down regulation of bone morphogenic protein 7 (BMP7) in OSMF tissues.\textsuperscript{52} Alterations of the APC and wild-type p53 tumor suppressor genes in OSMF may imply a risk for progression to oral cancer.\textsuperscript{53} Tumor necrosis factor-alpha (TNF-alpha), situated in the class III region of human leukocyte antigen (HLA), is a mediator with multiple functions, including the regulation of inflammatory reaction and transcriptions of collagen and collagenase. The high production allele, TNF2, was significantly lower among OSMF subjects.\textsuperscript{54} Increased levels of proinflammatory cytokines and reduced antifibrotic interferon gamma (INF-\textgamma) were demonstrated in patients with OSMF.\textsuperscript{55} In particular, the phenotype frequency of allele A6 of Major histocompatibility complex class I chain-related gene A (MICA) in subjects with OSMF was significantly higher and suggested a risk for OSMF.\textsuperscript{56} The number of high affinity rosette forming cells (HARFC) was found to be significantly decreased and levels of serum IgA, IgD and IgE were found to be elevated in OSMF. The enumeration of HARFC along with the estimation of serum levels of IgA, IgD, and IgE indicates that OSMF can be an intermediary stage in the malignant transformation of a normal cell.\textsuperscript{57}

Following leukocyte activation, the interaction between leukocyte integrin heterodimers and endothelial superfamily adhesion ligands results in a firm adherence of leukocytes to endothelium, leading to
leukocyte migration and homing to sites of mucosal inflammation consistently seen in OSMF.58

Role of infection
- HPV DNA, HSV DNA and EBV DNA were detected from patients with OSMF.59
- Role of H pylori in the etiology of mucosal inflammation, a condition that compounds the morbid state associated with OSMF was assessed using Rapid urease test (RUT) of plaque samples to estimate the H pylori bacterial load. A positive correlation exists between RUT reactivity and the frequency of mucosal inflammation.60
- The prevalence of oral yeast carriage in patients with OSMF as compared to the carriage with the normal individuals was assessed. The carriage of yeast in the OSMF group was not statistically significant compared with the control group. C. dubliniensis was isolated from the oral cavities of both OSMF patients and healthy individuals.51

Others
The serum beta carotene level was found to be lower in the patients with OSMF. When the values were compared between different disease stages, the maximum reduction of beta carotene was evident for Grade III OSMF, as compared with Grade I and II.62 A protective effect of wheat was observed in OSMF.63

Normal fibroblast cultures were incubated with areca nut alkaloids (arecoline, arecaidine). The cultures had a dose-dependent reduction in the proportions of phagocytic cells. On the other hand, corticosteroid used in the treatment of OSMF exhibited a dose-dependent enhancement in the proportion of phagocytic cells. Betel nut alkaloids (arecoline, arecaidine) inhibit fibroblast phagocytosis and this provides a mechanism for the development of OSMF.64 Immunohistochemical method to quantify the T lymphocyte, B lymphocyte and macrophage densities in the epithelium and sub epithelial connective tissue showed CD4+ to CD8+ lymphocyte ratio was 2.1:1. Increase in the number of T-lymphocytes and macrophages and a predominance of CD4 lymphocytes over CD8 lymphocytes was observed by Chiang CP et al.65 Plasma FDP is reported to be an early indicator of fibrin deposition. When the plasma Fibrin degradation products (FDP) increases, the fibrin deposited also increases. This strengthens the finding that OSMF is primarily a change of connective tissue causing excessive deposition of fibrin. This in turn leads to restriction of mouth opening.66

Conclusion
It is mandatory to have a thorough knowledge about the pathogenesis involved in the occurrence of OSMF as the disease has no gold standard management since any form of treatment given causes morbidity to the patient. Hence, unraveling various facts about the pathogenesis would ensure the oral physicians to formulate a standard treatment for the patients in need.

Statement of Clinical Relevance
1. This manuscript describes the past and present scenarios and concepts in the pathogenesis of Oral Submucous Fibrosis
2. An overview of different mechanism involved in pathogenesis of OSMF to date
3. A flow chart to summarize the concepts

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Flow Chart 1: A multifactorial model for the pathogenesis of oral submucous fibrosis.