Assessment of collagen and elastic fibres in various stages of oral submucous fibrosis using Masson's trichrome, Verhoeff vangieson and picrosirius staining under light and polarizing microscopy

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ABSTRACT

Background: Oral Submucous Fibrosis (OSMF) is a collagen related disorder seen in habitual betel-quid chewers. This is a high risk precancerous condition which clinically presents vertical palpable fibrous bands in buccal mucosa, generalized fibrosis of oral soft tissues with restricted mouth opening. Present study was undertaken to ascertain the changes occurring in collagen type I & III and elastic fibres in OSMF in relation to orientation, density and thickness.

Study Methods: The study was performed on 15 cases in each group such as Stage I OSMF, Stage II OSMF, Stage III OSMF and normal oral mucosa (NOM). The biopsied samples were routinely processed for paraffin embedding; stained with Hematoxylin and Eosin as well as special stains like Masson's Trichrome (MT), Verhoeff Van Gieson (VVG) and Picrosirius red (PSR) and examined under light and polarized microscope respectively. MT stain demonstrated all types of collagen fibers collectively while PSR stain under polarized microscopy demonstrated collagen type I and type III separately with enhanced birefringence. VVG stain demonstrated very fine black colored elastic fibers, thus changes taken place could be accurately ascertained with progression of the disease.

Results: Change in the orientation of collagen type I has been observed in stage II and stage III of OSMF from haphazard to parallel in relation to surface epithelium, while no change was noted in type III collagen fibres as well as elastic fibres in any stage of OSMF, and remained haphazardly arranged. Increased density of type I collagen was observed with increasing stage of OSMF from moderately dense to dense, while decrease in density of type III collagen was noticed in stage III than in stage II OSMF from moderately dense to sparse. The density of elastic fibres was decreasing from dense in stage I to sparse in stage III. Thickness of collagen type I was increasing with increasing grades while type III collagen and elastic fibres remained unchanged.

Conclusion: The alterations in orientation, density and thickness of collagen fibres and density of elastic fibres in various grades of OSMF contribute to the clinical presentation of trismus with progression of the disease.

Key Words: Collagen type I, Collagen type III, Elastic fibres, Masson's trichrome stain, Verhoeff Van Gieson stain, Picrosirius red stain

INTRODUCTION

Oral submucus fibrosis (OSMF) is a chronic debilitating disease of oral mucosa characterized by generalized fibrosis of the oral soft tissues which tend to present itself clinically as palpable fibrous bands.[1] The most common initial symptoms of OSMF are burning sensation of oral mucosa aggravated by spicy food, followed by either hypersalivation or dryness of the mouth.[2] It may also be preceded by ulceration or pain.[1] The hallmark of OSMF is that it affects most parts of oral cavity, pharynx and upper third of esophagus leading to dysphagia and progressive trismus due to rigid lips and cheeks.[3]

The overall prevalence rate of OSMF in India is about 0.2% to 0.5% and prevalence by gender varying from 0.2 to 2.3% in males & 1.2 to 4.57% in

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females. The age range of the patients with OSMF is wide which ranges between 20 to 40 years.[4]

The disease is most commonly found in youth as they are more attracted to commercially available areca nut products.[5] The alkaloids and flavonoids from arecanut stimulate proliferation of fibroblasts and collagen synthesis.[6] OSMF fibroblasts synthesize large amount of collagen compared to normal fibroblasts.[7] Thus in OSMF connective tissue changes are characterized by deposition of dense collagen fibers.[1] Moreover hyaline degeneration, fragmentation and elastic degeneration are characteristic observations with the progress of the disease.[8]

About 26 types of collagen fibers are identified so far depending on molecular structure. Of these collagen; type I is most abundant interspersed by type III in connective tissue.[9] Routine Hematoxylene and Eosin (H&E) and Masson's Trichrome stain (MT) demonstrate all types of collagen collectively. However Picrosirius red (PSR) stain under polarized microscopy demonstrate collagen type I and type III separately with enhanced birefringence. Collagen type I appear as closely packed thick fibers with intense birefringence of yellow / orange to red color and correspond to collagen fibers. However collagen type III appear as loosely packed thin fibrils which display a weak birefringence of green to greenish-yellow color that could be identified as reticular fibers. Thus characterization of collagen becomes specific and reliable with variable thickness and different color intensities of birefringence.[10, 11]

Normal mucosa is elastic, flexible and resilient. Elastic fibers are the major insoluble extracellular matrix assemblies that endow resilience to connective tissue permitting long range deformability.[12] However in OSMF oral mucosa shows reduced elasticity and flexibility with progress of disease because of deposition of excessive collagen.

Thus the purpose of this study was to ascertain the importance of orientation, density and thickness of collagen type I and type III and elastic fibers in various stages of OSMF.

MATERIALS AND METHODS

The present study included 15 NOM and 45 OSMF subjects which were divided equally in three groups as stage I, II and III following clinico-functional classification by Haider et al (2000) after obtaining written consent of the patient and institutional ethical committee clearance.

Further incisional biopsy was performed from buccal mucosa for the selected cases, fixed in 10% neutral buffered formalin and processed for paraffin embedding. 4 µm thick sections were obtained using semiautomatic microtome, stained with MT, VVG and PSR stains based on standard protocol, and observed under light and Polarized microscope respectively.[13] Thickness of collagen and elastic fibers was measured with the help of LYNX software (Lawrence & Mayo) in 10 randomly selected fields per sample without overlapping.

RESULTS

Present study revealed following results -

Orientation of collagen and elastic fibres:

The collagen fibres (MT stained) [Fig-1] and type I collagen (PSR stained) exhibited haphazard arrangement in NOM and stage I OSMF, while its orientation was changed and appeared parallel to the surface epithelium in stage II and III OSMF. However type III collagen (PSR stained) and Elastic fibers (VVG stained) did not show any alteration in various stages of OSMF from NOM and appeared haphazard (Table-1) [Figure-2]

Table-1: Pattern of Orientation of Collagen & Elastic fibers in relation to the surface epithelium in NOM and OSMF

	NOM (15)	**STAGE I** (15)	**STAGE II** (15)	**STAGE III** (15)
COLLAGEN IN MT	Haphazard	Haphazard	Parallel	Parallel
COLLAGEN TYPE I IN PSR	Haphazard	Haphazard	Parallel	Parallel
COLLAGEN TYPE III IN PSR	Haphazard	Haphazard	Haphazard	Haphazard
ELASTIC FIBERS IN VVG	Haphazard	Haphazard	Haphazard	Haphazard

Density of collagen and elastic fibres:

Type I collagen fibers (PSR stained) appeared moderately dense in NOM and stage I OSMF, while its density increased in stage II & stage III OSMF. [Figure-2]

Similarly density of Type III collagen fibers (PSR stained) appeared moderate in NOM, stage I & stage II OSMF but it was sparse in stage III OSMF. [Figure-2]

Moreover dense elastic fibers (VVG stained) were observed in NOM and stage I OSMF, which showed decrease in density with progress of OSMF and appeared moderately dense in stage II OSMF and sparse in stage III OSMF. (Table 2) [Figure-3]

Table-2: Comparison of density of Collagen & Elastic fibers in NOM and OSMF

DENSITY OF FIBERS	NORMAL (15)	STAGE I (15)	STAGE II (15)	STAGE III (15)
COLLAGEN TYPE I IN PSR	MOD DENSE	MOD DENSE	DENSE	DENSE
COLLAGEN TYPE III IN PSR	MOD DENSE	MOD DENSE	MOD DENSE	SPARSE
ELASTIC FIBERS IN VVG	DENSE	DENSE	MOD DENSE	SPARSE

Thickness of collagen and elastic fibres:

Measured thickness of collagen fibres (MT stain) [Fig-1] and type I collagen (PSR stained) was more in OSMF than NOM. [Fig-2] Moreover progressive increase in thickness was noticed with advancement of OSMF.

Type 3 collagen fibres [Fig-2] and Elastic fibres [Fig-3] showed little variation in thickness in NOM and various stages of OSMF. (Table 3)

Table-3: Comparison of thickness of Collagen fibres, Collagen type I, Collagen III & Elastic fibers in NOM and OSMF in µm

THICKNESS OF FIBERS	NORMAL (15)	STAGE I (15)	STAGE II (15)	STAGE III (15)
COLLAGEN FIBRES IN MT	1.1	2.5	3.9	8.7
COLLAGEN TYPE I IN PSR	1.9	4.1	7.1	11.7
COLLAGEN TYPE III IN PSR	1.8	1.9	2.0	1.68
ELASTIC FIBERS IN VVG	1.2	1.2	1.1	1.2

Figure-1: Photomicrograph Showing Orientation of Collagen Fibers In Relation To the Surface Epithelium in Various Stages of OSMF (MT)

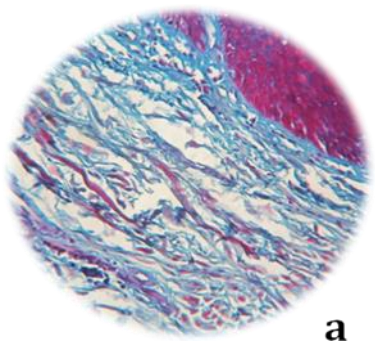
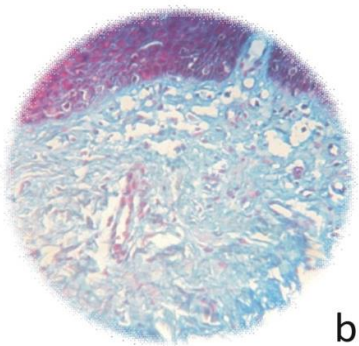
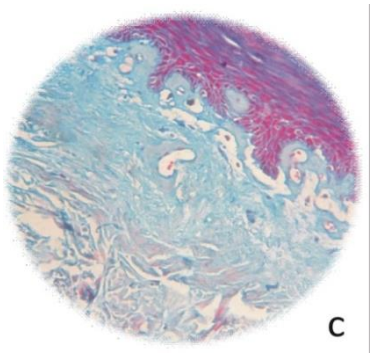
Stage I	Stage II	Stage III
 a	 b	 c

Figure-2: Photomicrograph Showing Orientation of Collagen Type I & Type III Fibers In Relation To the Surface Epithelium in Various Stages of OSMF (PSR)

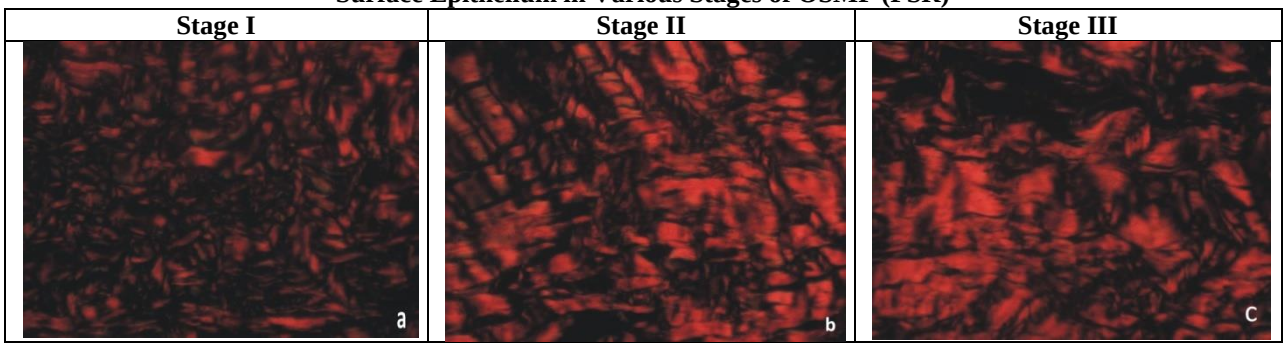
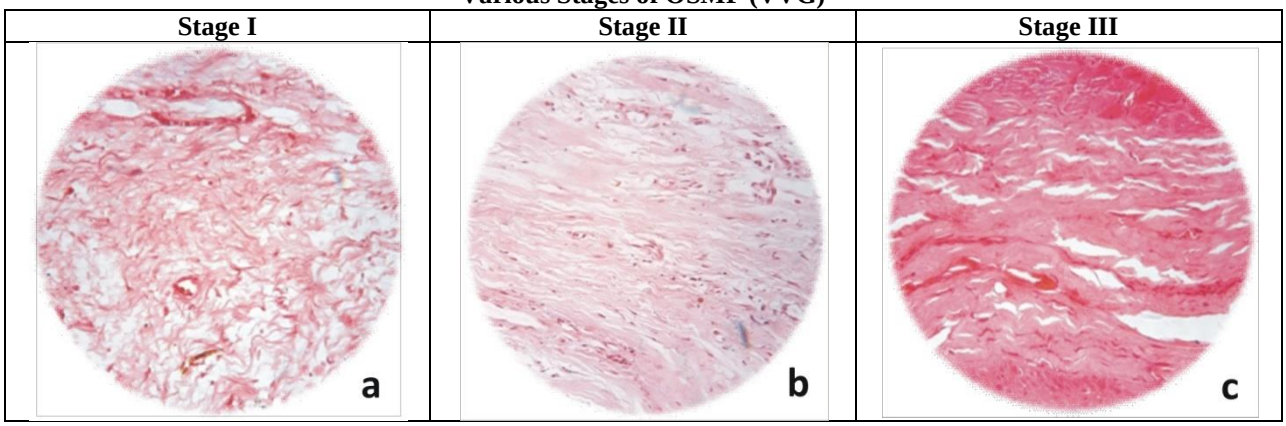


Figure-3: Photomicrograph Showing Orientation of Elastic Fibers In Relation To the Surface Epithelium in Various Stages of OSMF (VVG)



DISCUSSION

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 tissue. Various authors have agreed that pathological alteration in OSMF begin in the lamina propria and the epithelium responds only secondarily. Fibrosis and hyalinization extends into muscle bundle zone resulting into atrophy of the muscles. MT is a special stain which offers a simultaneous contrast color to the collagen fibers along with muscle fibers facilitating better visual discrimination between them.[8]

Collagen is the major structural element of the connective tissue which contributes to the stability and maintains structural integrity. It contributes to the entrapment, local storage and delivery of growth factors and cytokines and play an important role during organ development and tissue repair. So far 26 genetically distinct collagen types have been described. Type I collagen fibers form the bulk of subepithelial collagen while type III is intermixed with it.[9] PSR is the special stain for connective tissue especially for differentiating collagen subtypes.[11] It works on the principle that sulfonic group of sirius red- a strong cationic dye reacts with the basic groups present in collagen molecules. The elongated dye molecules are attached to collagen fibers in such a way that their long axis is parallel. This parallel relationship between dye and collagen molecules results in enhanced birefringence. The role of picric acid is to prevent the indiscriminate staining of non collagenous structures by sirius red.[2]

Enhanced birefringence of the fibers could be demonstrated by polarizing microscopy. Various colors exhibited by different types of collagen provide information regarding the type of the collagen with respect to its physical aggregation and morphological appearance. Thus these polarizing colors help in grading the severity of the disease.

Moreover it may serve as an excellent adjunct to electron microscopic study.[10]

Present study revealed haphazard orientation of collagen fibres as well as type I collagen in NOM and stage I OSMF, while in stage II and stage III most of them were arranged parallel to the surface epithelium. Type III collagen fibers showed haphazard orientation in NOM and all stages of OSMF. Contrasting to these findings Parveen S et al (2013) observed parallel orientation of thin (type III) and thick (type I) collagen fibers to the epithelium in all grades of OSMF and stated that the cause for these unidirectional or parallel alignments may be due to -

- Chronic stimulation of oral mucosa by irritation or as sequence of mechanical stress.
- Due to force generated by cell mediated gel contraction.
- Due to changes in the extracellular matrix imbalance production and degradation.[1]

Moreover Smitha BR et al (2013) explained that parallel orientation of collagen fibers to the epithelium in 68% in buccal mucosa and 78% in labial mucosa of OSMF subjects was due to their deposition in the direction of opening and closing movement of mouth.[2]

Density of collagen type I and type III appeared moderate in NOM as well as in stage I OSMF, however collagen type I appeared denser in stage II and stage III OSMF. Collagen type III appeared moderately dense in NOM and stage I and stage II OSMF, while they were sparse in type III OSMF. These findings show increased density of collagen type I with increasing stages of OSMF and was found to be statistically significant ($p<0.05$), while density of type III collagen appeared same in NOM and stage I & II OSMF, and reduced in type III OSMF. These findings are consistent with Parveen S et al (2013), Kamath VV et al (2013), Ganganna K et al (2012) and Ceena DE et al (2009).[1,11,14,15] Kamath VV et al has further explained the reduction in type III fibres to be on the basis of the compaction (removal of extracellular matrix substance) during progressive maturation of the fibres.[11]

In the present study average thickness of collagen fibers in stage I, III and III OSMF was 2.52 µm ± 0.19; 3.96 µm ± 0.50 and 8.7 µm ± 1.5 respectively while 1.11 µm ± 0.43 in NOM. Further assessment of average thickness of type I collagen under polarizing microscope revealed 4.15 µm ± 0.86; 7.18 µm ± 0.83 and 11.7 µm ± 1.0 in stage I, II and III respectively and 1.90 µm ± 0.72 in NOM. This demonstrates that there is great increase in thickness of type I collagen with increasing stages of OSMF and further it can be stated that polarized microscopy after PSR staining gives more precise measurement due to different pattern of birefringence. Ganganna K et al (2012), Ceena DE et al (2009) and Kamath VV et al (2013) too observed increase in thickness of collagen fibres with increasing grades of OSMF.[11,14, 15] Moreover in the present study average thickness of type III collagen in NOM, stage I and II OSMF appeared nearly same with slight reduction in stage III OSMF. However Kamath VV et al (2013) observed variable results for type III collagen.[11]

The normal lining mucosa shows elastic fibers interlacing in all directions and provide elasticity. A VVG special stain demonstrates very fine black colored elastic fibers. The changes taken place in elastic fibers thus could be accurately ascertained by VVG staining.[13]

The present study demonstrated haphazard orientation of elastic fibers in all directions in NOM and all stages of OSMF which was statistically significant (Kruskal- Wallis Test).

Moreover the density of elastic fibres in NOM and stage I OSMF appeared more, which was moderate in stage II and sparse in stage III OSMF. These findings demonstrated that density of elastic fibers decreases with progress of disease and results were statistically significant ($p<0.05$).

However observed average thickness of elastic fibers in stage I, II and III OSMF was 1.20 µm ± 0.08, 1.19 µm ± 0.07 and 1.2 µm ± 0.08 respectively while in NOM it was 1.2 µm ± 0.08; which suggests that there is no much change in thickness of elastic fibres in OSMF when compared to NOM.

From these observations an assumption can be made that though thickness and orientation of elastic fibres remain almost same in all stages of OSMF as NOM the decreased density of elastic fibers could be playing significant role in reduction of elasticity of mucosa leading to decreased mouth opening.

CONCLUSION

It can be inferred that collagen fibers and elastic fibers show minimal alternation in early stages. Once the disease progress from early stages and patients continues with arecanut chewing habit the fibrotic changes in connective tissue accelerates in severity. Unfortunately even after cessation of the causative habit, all the clinical and histologic features of the disease persists. Moreover collagen fibers change

their orientation and become unidirectional and arranged parallel to the epithelium which can be correlated with the direction of force.

Further polarized microscopic study strongly suggests that type I fibers represented predominantly as orange red- red colored fibrils and are deposited in excess as severity of disease increases whereas type III collagen fibers appear fine and show week greenish yellow birefringence.[14] These fibers decrease with increase in severity which can be explained as removal of extracellular matrix substance during progressive maturation of fibers.[1]

However elastic fibers reduce with increase of severity of disease. This can be correlated further with decrease or loss of elasticity of mucosa with progression of disease.

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