Original Article

Polarized microscope assisted study of collagen fibres in Oral Submucous Fibrosis and their clinicopathologic correlation: An in-vitro study

Ameena Singh^{a,*}, Sukumar Singh^b, Susmita Saxena^c, Sanjeev Kumar^b, Akanksha Banga^a, Nitin Purohit^b^a Department of Oral Pathology and Microbiology, ITS-CDSR, Muradnagar, Ghaziabad, India^b Department of Oral and Maxillofacial Surgery, ITS-CDSR, Muradnagar, Ghaziabad, India^c Department of Oral Pathology and Microbiology, ESIC Dental College, Rohini, New Delhi, India

ARTICLE INFO

Article history:

Received 23 November 2016

Received in revised form 18 June 2017

Accepted 8 July 2017

Available online 14 July 2017

Keywords:

Collagen

Oral submucous fibrosis

Picrosirius red

ABSTRACT

Background: The present study was aimed to evaluate and analyze orientation and birefringence pattern of collagen fibers histopathologically in different grades of OSMF using the special stain (Picrosirius red) under polarizing microscope and relate these factors with the severity of OSMF.

Materials and Method: 45 confirmed cases of OSMF were taken from the archives. The selection of clinical cases was done according to classification by J.N Khanna and Andrade (1999). These cases were correlated histopathologically as per the criteria given by Pindborg JJ and Sirsat S.M (1966). The sections were stained with H & E and Picrosirius red stains and assessment was done accordingly. 10 cases of normal mucosa were taken as control.

Results: In clinical grade I, 60% of the cases showed histopathological grade II, while 53.4% cases in clinical grade II correlated with histopathological grade III and 33.4% of clinical grade III cases showed histopathological grade IV. Upon application of Fisher's exact test a significant P value <.01 was obtained suggesting no statistical significant correlation between these two parameters. However, the orientation pattern revealed a parallel orientation as OSMF advanced. Also as the severity of the disease increased, there was a gradual shift from green- greenish yellow colour of the fibres and a shift to orange red-red colour.

Conclusion: Upon correlation of clinical grades with histopathological grades, no statistically significant difference was found. Birefringence pattern of collagen revealed similar findings as other studies in case of clinical grades but histopathological grades showed a contrast finding.

© 2017 Craniofacial Research Foundation. All rights reserved.

1. Introduction

The first ever mention of Oral Submucous Fibrosis (OSMF) was by SUSHRUTA in his classification of mouth and throat disorders in which he called it 'VIDARI'. He described features of progressive narrowing of mouth, depigmentation of oral cavity and pain on eating food associated with the condition.¹ OSMF has also been described as 'Atrophica idiopathica tropica mucosae oris' (Schwartz, 1952). Joshi (1953) coined the term 'Submucous fibrosis of the palate and pillars'. Other names that have been suggested are 'Diffuse oral submucous fibrosis' (Lal L, 1953), 'Idiopathic

scleroderma of the mouth' (Su .I.P. 1954), 'Idiopathic palatal fibrosis' (Rao A.R.N, 1962) and 'Sclerosing stomatitis' (Behl P.N, 1962).¹

OSMF is defined by Pindborg¹ as "an insidious chronic disease affecting any part of the 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 fibro elastic change of the lamina propria, with the stiffness of the oral mucosa, trismus and inability to eat"¹.

The disease mainly occurs in Indian subcontinent and affects 0.2–1.2% of the urban population. It has a female preponderance and mostly presents in the middle life, mean age being 43 years. Initial signs & symptoms of the disease are inability to tolerate spicy food and blanching of the oral mucosa usually symmetrically.

* Corresponding author.

E-mail address: draminasingh@gmail.com (A. Singh).

As the disease progresses, thick inelastic fibrous bands appear in the sub mucosal layer of the mucosa, faucial pillars, floor of mouth and tongue. In advanced cases, uvula becomes atrophic, the mobility of soft palate and tongue is markedly reduced and there may be atrophy of tongue papillae. In most advanced cases, it can lead to difficulty in mastication, deglutition and phonation. It is also associated with epithelial dysplasia and there is an increased risk of carcinomatous changes, hence it is considered to be a precancerous condition.² Malignant transformation of OSMF has been reported to be around 7.6%.²

OSMF is believed to have a multifactorial etiology. A number of factors that trigger the disease process are mainly areca nut (Betel nut) chewing, ingestion of chillies, nutritional deficiencies and genetic alterations. Constant immunological abnormalities in patients with OSMF are found which could have implication for the malignant transformation of the lesions.³

Betel quid (BQ) is the fourth most common substance of misuse in the world after tobacco, alcohol and caffeine. The areca nut (Betel nut) is a component of Betel quid (BQ). The flavonoid content of areca nut has direct influence on collagen metabolism causing OSMF. The Areca nut is the seed of the areca palm (*Areca catechu*). It is not a true nut but rather a drupe and is commercially available in dried, cured and fresh forms.⁴

Areca nut is a mild stimulant, causing a hot sensation in the body, heightened alertness and sweating, although the effects vary from person to person. The areca nut contains tannin, Gallic acid, a fixed oil gum, a little terpeneol, lignin, various saline substances and three main alkaloids: Arecoline, Arecaidine, Guacine which have vasoconstricting properties.⁴ Areca nuts also have been shown to have a high copper content, and chewing areca nuts for 5–30 minutes significantly increases soluble copper content in oral fluids which might account for fibrosis in OSMF.⁵

The quantitative determination of collagen is time consuming and not very sensitive. To overcome this problem, Picrosirius red stain may be used as an effective stain in assessment of collagen fibres in OSMF. Sirius red is a strong cationic dye which stains collagen by reacting via sulfonic acid groups with basic groups present in the 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 molecules results in enhanced birefringence. The role of picric acid is to prevent the indiscriminate staining of non-collagenous structures by Sirius red.^{6,7,8}

This study was therefore, designed to evaluate and analyze the orientation and birefringence pattern of collagen fibers histopathologically in different grades of OSMF using Picrosirius red stain under polarizing microscope and to relate these factors with the severity and progression of OSMF.

2. Materials and method

The present study was carried out on formalin fixed, paraffin embedded tissues of histopathologically diagnosed cases of Oral submucous fibrosis retrieved from the archives of the Department of Oral Pathology. Clinical details of the patients were obtained from the records of Department of Oral Medicine and Radiology. Cases were clinically graded as per the criteria given by Khanna and Andrade.⁹ Histopathological grading of OSMF cases was done as per the criteria given by Sirsat and Pindborg.¹⁰

Of the selected 45 cases, 15 cases each were selected in three clinical grades. For each case, 2 sections were made, of which one section was stained with routine H&E stain, used to grade the cases histologically, while the other section was stained with special stain Picrosirius red to assess the collagen fibers orientation and birefringence pattern, thus analyzing the maturation of collagen. 10 cases of normal buccal mucosa were selected as controls for

comparison. For H & E and Picrosirius stains the methods were followed as suggested by Suvarna et al.¹¹

The Picrosirius red stained sections were viewed using polarized microscopy. In bright field the collagen fibres appeared red on a pale yellow background while nuclei were gray to brown in color. However, when examined through crossed polars, the largest collagen fibers were bright yellow to orange, and the thinner ones yellowish green to green. Upon application of Fisher's exact test a significant p value <.01 was obtained.

3. Results

Upon correlation of clinical grades with histopathological grades, it was noted that out of 15 clinical grade I cases, 60% of the cases showed histopathological grade II, similarly in clinical grade II 53.4% of the cases were in histopathological grade III. In clinical grade III, 53.4% of cases were in histopathological grade III and 33.4% were in histopathological grade IV (Fig. 1).

A comparison of the orientation of collagen fibers in each clinical grade of OSMF was done and it was found that out of total cases in clinical grade I, 13.4% cases showed parallel orientation, 40% cases showed non-parallel orientation, and a mixed orientation was observed in 46.7% cases. In clinical grade II, 53.4% cases showed parallel orientation, 26.7% revealed a non-parallel orientation and mixed orientation was noted in 20% cases. In clinical grade III, parallel orientation was noted in 33.4% cases, non-parallel orientation was noted in 26.7% cases and mixed orientation was noted in 40% cases (Fig. 2).

However an overall comparison of orientation of collagen fibers in different histopathological grades was done and the results have been tabulated in Table 1. As regards to the birefringence pattern of collagen fibers in each clinical grade of OSMF a comparison was done. It was noted that out of 15 cases in clinical grade I, 33.4% cases showed yellow orange fibers. In clinical grade II, 13.4% cases showed yellow orange fibers and 13.4% cases showed red fibers. In clinical grade III, 26.7% cases showed red fibers (Fig. 3).

Similarly an analysis of birefringence pattern in each histopathological grades was done and it was noted that out of 12 cases in histopathological grade II, 41.7% cases showed yellow orange fibers; whereas in histopathological grade III, out of 21 cases, 23.8% cases showed yellow orange fibers; and in histopathological grade IV, out of 12 cases, 16.7% cases showed (Fig. 4). The varying birefringence pattern of collagen fibres in oral submucous fibrosis has been depicted in Fig. 5.

4. Discussion

Prevalence of OSMF has been on the rise, which at present is attributed to the use of arecanut and its products in variety of ways. Lack of awareness, aggressive marketing strategies of these products and failure to impart primary preventive steps have also contributed in the spread of the disease in a considerable manner among the rural and urban younger population. This is evident from the fact that the number of OSMF patients approaching for medical care in very early stages of the disease is probably a minuscule of total number of patients affected by the disease. The gravity of the problem can be assessed by the fact that by 1998, total estimated number of patients in India affected by the disease was approximately 2 million cases,¹² which is an eight fold increase in number, when compared to the reports by JJ Pindborg.¹³ Studies on collagen in OSMF has been a major focus in research as arecoline, the main alkaloid constituent of the arecanut has been reported to induce excessive collagen synthesis.⁷ Studies have shown that arecoline induces collagen formation, demonstrates cytotoxicity and also stimulates double stranded nucleic acid synthesis and cell- morphology change.^{9,14}

Fig 1. Co-relation of clinical and histopathological grades in oral submucous fibrosis.

Fig. 2. Orientation of collagen fibres in different clinical grades of oral submucous fibrosis.

Various studies on collagen fibers using polarizing microscope have been carried out by Ceena D,¹⁵ Smitha BR,¹⁶ Ganganna P,¹⁷ Gannepalli A¹⁸ and Parveen S.¹⁹ In OSMF, connective tissue changes are characterized by a deposition of dense collagen fibers. The

Picrosirius red stain is found to be superior to other stains. It stains fine collagen fibers more intensely and increases their birefringence spectacularly. Also, Sirius red stains are more stable and do not fade easily when compared to other stains.¹⁵

The advantage of using Picrosirius red stain is that the properties of enhanced birefringence demonstrated by the fibres could be exploited to study the various colors exhibited by different types of collagen which provides the researchers information regarding the quality of the collagen with respect to its physical aggregation and morphological appearance. The equipment and materials used are inexpensive; the technique is relatively easier to perform and is reproducible. Moreover the polarizing colors itself helps in grading the severity of the disease.^{6,7}

Table 1

Orientation of collagen fibres in different Histopathological grades of Oral Submucous Fibrosis.

S.NO	ORIENTATION	H/P GRADE II	H/P GRADE III	H/P GRADE IV
1	PARALLEL	2 (16.7%)	9 (42.8%)	4 (33.4%)
2	NON- PARALLEL	6 (50%)	5 (23.8%)	3 (25%)
3	BOTH	4 (33.4%)	7 (33.4%)	5 (41.7%)
TOTAL		12	21	12

Fig. 3. Birefringence pattern of collagen fibres in different clinical grades of oral submucous fibrosis.

Fig. 4. Birefringence pattern of collagen fibres in different histopathological grades of oral submucous fibrosis.

Fig. 5. Picrosirius stain showing varying birefringence of collagen fibres in oral submucous fibrosis. Greenish yellow(a), yellow(b), yellow orange(c), orange red(d) and red(e) birefringence (Picrosirius stain, 100×). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The OSMF cases in our study showed no noteworthy differences when compared with previous studies, while considering overall histopathological features. The histopathological features of the connective tissue in OSMF are quite characteristic with occurrence of dense collagen fibres, juxtaepithelial hyalinization, dilated blood vessels in early stages and constricted vessels in advanced stages. The fibroblasts were plump and increased in number in early stages whereas their numbers were found to decrease in advanced stages of the disease,^{18,19} similar results were observed in the present study also.

In the present study, collagen fibres appeared thin in early stages i.e. grade II of the disease according to Sirsat and Pindborg,¹⁰ especially in the superficial lamina propria. Few cases in these stages showed evidence of hyalinization of the fibres. Hyalinization present was predominantly restricted to subepithelial zone. As the disease progressed, the fibres appeared thicker and many sections showed hyalinization of the fibres even at deeper submucosal regions, and diminished cellular constituents. We also noted an increased fibroblast population in mild form of OSMF when compared to the controls and severe cases; these findings are in accordance with the study by Sirsat SM & Pindborg JJ.¹⁰

Our study revealed homogenization and hyalinization of collagen fibres in few cases which failed to reveal well formed, well defined, discernible fibres in the superficial lamina propria. The deeper layers were showing thicker fibres which were more discernible. This group represented grade III i.e. moderately advanced stage in our study. However in few cases, even the deep connective tissue revealed intense fibrosis in and around the minor salivary glands and muscle tissue. These cases revealed a few patent blood vessels and the capillaries appeared to be almost obliterated in superficial lamina propria. Such cases were categorized into grade IV i.e. advanced stages of the disease.^{10,15} With regard to the overlying stratified squamous epithelium, many cases showed atrophy of the epithelium in moderately advanced and advanced stages. In contrast to studies done by Ceena D,¹⁵ Smitha BR,¹⁶ Parveen S,¹⁹ in our research an equal number of patients were selected in all the three clinical grades of OSMF and a correlation between clinical and histopathological grades was done. Results revealed that out of 45 OSMF subjects, 12 (26.7%) cases were histopathological grade I, 21 (46.7%) cases were histopathological grade II and 12 (26.7%) were histopathological grade III. This however revealed that there was no correlation was found in clinical and histopathological grading.

The findings with regard to orientation of collagen fibres are in accordance to studies done by Ceena D,¹⁵ Smitha BR,¹⁶ Parveen S,¹⁹ which showed few parallel fibers, in the upper half of connective tissue in grade I OSMF. In different literature studies, in histopathological grade II OSMF cases, a parallel orientation of collagen fibers was observed. The lower half showed thick collagen fibers. In histopathological grade III, OSMF cases the upper and lower half of connective tissue revealed collagen bundles that are tightly packed.^{15,16,18,19}

In the present study when, the clinical grades of OSMF were analyzed for the birefringence pattern, it was noted that yellow orange birefringence was predominant in clinical grade I. In clinical grade II, green yellow and yellow orange birefringence pattern was predominant. While clinical grade III showed a predominance of red birefringence. These findings are in agreement with studies by Ceena D, Ashalata G, Parveen S et al.^{15,18,19}

Upon analysis of birefringence pattern in histopathological grades of OSMF, it was noted that yellow orange birefringence was noted in histopathological grade I and II. In histopathological grade III, both green and yellow orange birefringence was noted. However histopathological grades were showing results in contrast to studies done by Ceena D,¹⁵ Smitha BR¹⁶ et al, Ashalata G¹⁸, and Parveen S.¹⁹

Junquiera L.C.U et al.²⁰ stated that under polarization microscopy using Picrosirius red stain, collagen type I shows up as thick, strongly birefringent yellow or red fibres while collagen type III appears as thin, weakly birefringent greenish fibres that could be characterized as reticulin fibers. Collagen type II does not form fibres and displays a weak birefringence with variable colors. It was noticed that color and intensity of birefringence depends on the collagen fibre thickness. This holds true for the present as well as previous studies done by Ceena D, Smitha BR et al, Ashalata G, Parveen S.^{15,16,18,19}

The reasons for parallel alignment of collagen fibers may be due to chronic stimulation of oral mucosa by irritation or as sequence of mechanical stress where fiber orientation with the direction exerted on them like in scars and keloids, also due to forces generated by cell-mediated gel contraction, or due to changes in the extracellular matrix imbalance production and degradation.¹⁹

Difference in color patterns of collagen fibers could be due to various growth factors and cytokines that cause proliferation of fibroblasts and extracellular matrix resulting in formation of thick mature collagen. As collagen matures, the change in proteoglycan content of fibers causes dehydration of the fibers thereby increasing the diameter of collagen fibers. The color may be due to the tight packing of collagen fibers. Hence, there is an increase in intensity of birefringence and change in the polarizing colors.²¹ As the connective tissue changes in OSMF precedes the epithelial changes, it may be considered that the connective tissue changes will give an earlier implication of OSMF than the epithelial changes as the basic defect lies in the collagen synthesis and degradation.¹⁷

In the present study the correlation between clinical and histopathological grades was analyzed. When each clinical grade was evaluated individually, a correlation was observed between the two parameters. However, overall analysis of the total sample size revealed no statistically significant correlation between the clinical and histopathological grades of OSMF. Study also underlines the variation of orientation and birefringence patterns in different histopathological grades, which depict the maturation of collagen as OSMF advances.

5. Conclusion

OSMF is indeed one of the classic 'Disease of civilization' with large difference seen between races, geographic areas and individuals at different levels in both prevalence and the degree to which it transforms into malignancy with continuation of habit. When the correlation between clinical and histopathological grades was analyzed in each clinical grade individually, a correlation was observed between the two parameters. Study likewise emphasizes on the variations of orientation in OSMF where a parallel orientation is seen as OSMF advances.

Birefringence patterns in different histopathological grades depend on tight packing of collagen, and alignment of fibers, which depict the maturation of collagen as OSMF progresses showing a green to yellow and red birefringence with the increase in severity of OSMF. The study has its limitations as the sample size being small and also less number of available cases in clinical grade IV/advanced cases of OSMF. Further no histopathological features were evident in any of the sections which could be categorized under very early histopathological stage of OSMF. Thus for future prospective, a larger sample size has to be analyzed to recognize the precise patterns of OSMF. Also, Picrosirius red stains very thin collagen fibers in comparison to other collagen stains. Therefore, further research must aim at ultra-structural features of connective tissue in different stages of OSMF to arrive at a substantial conclusion regarding the maturation pattern of collagen fibers.

Conflict of interest

The authors have none to declare.

References

1. Pindborg JJ, Sirsat SM. Oral submucous fibrosis. *Oral Surg Oral Med Oral Pathol*. 1966;22:764–769.
2. Pillai R, Balaram P, Reddi KS. Pathogenesis of OSMF. *Cancer*. 1992;69(8):2011–2020.
3. Rajlalitha P, Vali S. Molecular pathogenesis of OSMF – a collagen metabolic disorder. *J Oral Pathol Med*. 2005;34:321–328.
4. Murthi PR, Bhosle RR, Gupta PC, Daftary DK, Pindborg JJ, Mehta S. Etiology of OSMF with special reference to the role of areca nut chewing. *J Oral Pathol Med*. 1995;24:145–152.
5. Jaydeep K, Pillai R, Kannan S, Mathews B, Nair MK, Menon VP. Serum levels of copper, zinc, iron & ceruloplasmin in oral leukoplakia and squamous cell carcinoma. *J Exp Clin Cancer Res*. 1997;16(3):295–300.
6. Junqueira LCU, Bignolas G, Brentani RR. A simple and sensitive method for the quantitative estimation of collagen. *Analytical Biochemistry*. 1979;94:96–99.
7. Junqueira LCU, Bignolas G, Brentani RR. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochemical journal*. 1979;11:447–455.
8. Radhika T, Sekaran P, Narasimhan M. Qualitative analysis of collagen fibers in oral submucous fibrosis using picrosirius red stain and polarising microscope. *J Clin Diagn Res*. 2016;10(February(2)):ZC04–ZC07.
9. Khanna JN, Andrade NN. Oral submucous fibrosis: a new concept in surgical management –Report of 100 cases. *Intl J Oral Maxillofac Surg*. 1995;24:433–439.
10. Sirsat SM, Pindborg JJ. Subepithelial changes in oral submucous fibrosis. *Acta Path Microbiol Scand*. 1967;70(2):161–173.
11. Bancroft's Theory and Practice of Histological Techniques, 7th Edition. In: Kim S. Suvama, Christopher Layton, John D. Bancroft, (Eds.) 2012; 654.
12. Shah N, Sharma PP. Role of chewing & smoking habits in etiology of OSMF: a case control study. *J Oral Pathol Med*. 1998;27:475–479.
13. Pindborg JJ, Bhosle RB, Murthi PR, Gupta PC, Daftary DK, Mehta FS. Incidence and early forms of oral submucous fibrosis. *Oral Surg*. 1980;185(1):40–44.
14. De Waal J, et al. The fibroblast population in oral submucous fibrosis. *J Oral Pathol Med*. 1997;26:69–74.
15. Ceena DE, Bastian TS, Ashok L, Annigeri RG. Comparative study of clinicofunctional staging of oral submucous fibrosis with qualitative analysis of collagen fibers under polarising microscopy. *Indian J Dent Res*. 2009;20:271–276.
16. Smitha B, Donoghue M. Clinical and histopathological evaluation of collagen fiber orientation in patients with oral sub mucous fibrosis. *J Oral Maxillofac Pathol*. 2011;15(2):154–160.
17. Ganganna P, Shetty P, Shroff SE. Collagen in histologic stages of oral submucous fibrosis: a polarizing microscopic study. *J Oral Maxillofac Pathol*. 2012;16(2):162–166.
18. Gannepalli A, Baghirath PV, Krishna AB, Kumar PU, Quantitative Tom A. qualitative analysis of collagen in oral submucous fibrosis. *J Dr NTR Univ Health Sci*. 2012;1(2):99–105.
19. Parveen S, Ahmed SA, Tanveer S. A study on orientation of collagen fibres in oral submucous fibrosis. *Intl J of Sci Res Pub*. 2013;3(3):1–4.
20. Junqueira LC, Montes GS, Martins JE, Joazeiro PP. Dermal collagen distribution: a histochemical and ultrastructural study. *Histochemistry*. 1983;79(3):397–403.
21. Tsai CC, Ma RH, Shieh TY. Deficiency of collagen and fibronectin phagocytosis by human buccal mucosa fibroblasts in vitro as a possible mechanism for oral submucous fibrosis. *J Oral Pathol Med*. 1999;28:59–63.