ORIGINAL RESEARCH

Analysis of Polarization Colors and Collagen Fiber Thickness in Oral Submucous Fibrosis: Correlation with Different Chewing Habits

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Abstract

Introduction: Picrosirius red staining, combined with polarizing microscopy, enables the assessment of both quantitative and qualitative changes in collagen fibers in oral submucous fibrosis (OSMF), providing valuable insights into the degree of fibrosis and progression of the disease.

Aim: A qualitative analysis of collagen fibers across various histopathologic stages of OSMF was performed using Picrosirius red (PSR) stain and polarizing microscopy, with emphasis on the effect of three distinct chewing habits.

Materials and methods: A total of 78 OSMF cases were categorized into three groups based on chewing habits: areca nut alone, betel quid (with or without tobacco), and commercial areca products, with 26 cases per group. In each group, cases were selected from histopathological stages I to IV. Patients in stages I and II were grouped into Group I (39 cases), while those in stages III and IV formed Group II (39 cases). Collagen fiber thickness and polarization colors were evaluated using PSR staining and polarized microscopy, assessing variations across different chewing habits.

Results: A comparison of fiber thickness and color between Group I and Group II OSMF cases revealed statistically significant differences in both parameters. However, no significant differences in fiber thickness or color were observed across different chewing habits within Group I and Group II.

Conclusion: In this study, collagen fiber thickness increased progressively with the severity of the disease, and the polarization colors shifted from greenish-yellow to orangish-red and then to red as the disease advanced. The various chewing habits did not affect fiber color or thickness.

Key words: oral submucous fibrosis, areca nut, Picrosirius red stain, polarizing microscopy

Introduction

Oral submucous fibrosis (OSMF), a well-recognized oral potentially malignant disorder, most commonly seen in the Indian subcontinent has mainly been attributed to the use of areca nut and its products. With the increased use of these preparations, prevalence of OSMF is on the rise among the younger age group.[1,2] The overall prevalence of OSMF is about 4.47% worldwide and 6.36% in India.[3,4]

Areca nut chewing, in any form, is recognized as the primary causative factor OSMF. This condition predominantly affects males aged 20 to 40 years and can involve most areas of the oral cavity as well as the upper third of the esophagus.[5] Clinically, patients often present with symptoms such as a burning sensation in the mouth, excessive salivation, or xerostomia. As the condition progresses, the oral mucosa becomes rigid and opaque, with the formation of fibrous bands in the buccal mucosa, soft palate, lips, and tongue.[2,5] Histopathologically, OSMF is characterized by epithelial atrophy, loss of rete ridges, and sometimes epithelial atypia. The lamina propria shows subepithelial hyalinization, extensive col-

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lagen deposition, and degenerative changes in the underlying muscle tissue.[2,6]

The van Gieson and Masson trichrome histochemical stains are commonly used to visualize collagen in paraffin-embedded tissue sections. However, these stains do not differentiate between collagen types I and III.[7] Additionally, they may fail to adequately stain thin fibers, such as collagen type III, potentially leading to an underestimation of the tissue's collagen content.[8] In contrast, picrosirius red (PSR, also known as Sirius red) staining is regarded as the gold standard for detecting and quantitatively assessing collagen in histological sections of both normal and pathological tissues.[8,9]

Polarized light microscopy is used for analyzing and identifying optically anisotropic materials. The system consists of two polarizing elements: the polarizer, positioned between the light source and object plane, converts unpolarized light into plane-polarized light. The second element, the rotatable analyzer, is placed between the objective and eyepiece. In most cases, the polarizer and analyzer are "crossed," meaning their polarization directions are perpendicular. The polarizer restricts light to a specific orientation before it passes through the sample, where the internal structure of the material alters the light. When the analyzer is perpendicular to the polarizer, no light reaches the eyepiece, creating a dark field. Isotropic materials remain dark, while birefringent materials, which can modify polarized light, appear bright. Many substances, such as crystals, fibers, pigments, lipids, proteins, bone, and amyloid deposits, exhibit birefringence, allowing them to be visualized in this manner.[10,11]

When tissue sections stained with picrosirius red (PSR) are examined under polarized light microscopy, a complex array of colors corresponding to different collagen types is observed. This multicolored presentation is valuable for identifying and analyzing the distinct distribution patterns of structurally different collagen types. PSR is an anionic dye that binds with high specificity to the basic amino acids in collagen molecules due to its sulfonic acid groups.[12] Collagen exhibits birefringence, a property primarily attributed to the presence of acid mucopolysaccharides in the ground substance.[13] Picrosirius red dyes are elongated, birefringent molecules that, when bound within the upper groove of well-oriented eosinophilic collagen molecules, align parallel to the long axis of each collagen fiber. This alignment significantly enhances the inherent birefringence, which results from the uniform distribution of collagen fibers.[14]

Under polarized light, fibers stained with picrosirius red (PSR) display a spectrum of colors, which vary based on the fiber size, packing density, and the resulting orientation of the collagen fibers.[13] When visualizing PSR-stained tissues, thin collagen fibers (type III) typically appear green to greenish-yellow, while thicker collagen fibers (type I) range in color from yellowish-orange to orange-red. The green to greenish-yellow hue of both thin and thick fibers indicates that the collagen is loosely packed, whereas the orange-red coloration suggests tightly packed collagen fibers.[15] The predominance of green to greenish-yellow coloration in both thin and thick fibers suggests that the collagen molecules are loosely packed and may consist of procollagens, intermediates, or pathological collagen, rather than the tightly packed fibers characteristic of normal collagen.[11] Therefore, the combination of PSR staining and the enhancement of birefringence provides a simple, specific, and sensitive method for localizing collagen in tissue sections.[16,17] In the present study, a qualitative analysis of collagen fibers across different histopathologic stages of OSMF was conducted using PSR stain and polarizing microscopy, with a focus on three different chewing habits. Additionally, a clinicopathological analysis of these cases was performed.

Materials and Methods

OSMF cases were retrieved from the archives of the Department of Oral Pathology over the past two decades. Cases lacking adequate clinical and/or histopathological documentation were excluded from the analysis. A total of 78 cases were subjected to clinical and histopathological analysis in this study. Clinical data, encompassing parameters such as age, gender, lesion location, substance use habits (including areca nut consumption, commercial areca nut product usage, betel quid with or without tobacco, alcohol consumption, and smoking), habit frequency (number of times per day), habit duration (years of use), presenting symptoms, clinical staging, and any pertinent details, were methodically recorded and documented for the study. Histopathological grading, epithelial changes, and malignant transformation if present were recorded from the previous histopathological reports for subsequent analysis.

OSMF cases were initially categorized histopathologically using Pindborg's grading system into four stages: very early

Table 1: Association between clinical staging and histopathological staging

	Histopathological Staging						
		Stage I	Stage II	Stage III	Stage IV	Total	
Clinical Staging	Stage 1	5	6	1	0	12	Fisher's exact $\chi2 = 14.086$ p= 0.060, not significant
	Stage 2	11	12	15	11	49	
	Stage 3	2	2	7	4	15	
	Stage 4	0	1	0	1	2	
Total	18	21	23	16	78		

(Stage I), early (Stage II), moderately advanced (Stage III), and advanced (Stage IV).[18] For the purpose of comparative analysis, these were further grouped into two broader categories: Group I, comprising cases classified as Stage I and II (very early and early stages), and Group II, consisting of cases classified as Stage III and IV (moderately advanced and advanced stages). Each group consisted of 39 cases, which were further stratified into three sub groups of 13 cases each, representing distinct chewing habits-areca nut alone, betel quid (with or without tobacco), and commercial areca nut products to ensure equal representation across the different habit patterns.

One tissue section of each sample was stained with haematoxylin and eosin stain and the other with PSR stain. Haematoxylin and eosin stained sections were analyzed under light microscope following which histopathological staging was reconfirmed. PSR stained sections were examined under a polarized microscope to assess the thickness and polarization colors of collagen fibers within the connective tissue. In normal mucosa, collagen fibers generally range in thickness from 1 to 12 µm. Fibers measuring 5 µm or less were designated as thin, while those exceeding 5 µm were classified as thick based on the previous study.[17] For analysis, five high-power fields (100× magnification) were randomly selected from the sections. In each section, twenty-five clearly distinguishable fibers were randomly selected from both the subepithelial and deeper connective tissue regions for analysis. Fiber thickness was measured, and each case was categorized as having predominantly thin fibers (5 µm or less) or thick fibers (greater than 5 µm). Additionally, polarization colors for each case were recorded and classified as green to greenish-yellow (G-GY), yellowish-orange (YO), or orangish-red to red.

Statistical analysis was conducted using Fisher's exact test, with a p-value of < 0.001 considered indicative of statistical significance for comparisons of fiber thickness and polarization color between samples from Group I and Group II.

Table 2: Comparison of fiber thickness between Group I and Group II

		Group I	Group II	Total	Significance
Fibre thickness	Thin	36	8	44	$\chi2$ =40.877 p<0.001, significant
	Thick	3	31	34	
Total		39	39	78	

Table 4: Association of fibre thickness with habit in Group I

		Group I				
		Areca nut	Betel quid	Commercial areca nut products	Total	Fisher's exact $\chi2$ =0.434 p=1, not significant
Fibre thickness	Thin	12	12	12	36	
	Thick	1	1	1	3	
N	13	13	13	39		

Results

The study included a total of 78 cases of OSMF, confirmed through both clinical and histopathological evaluations. A notable male predominance was observed, with 72 cases (92.3%) being male and 6 cases (7.7%) female, resulting in a male-to-female ratio of 12:1. The most prevalent clinical manifestation observed was burning sensation, which was reported in 35 cases (44.9%), followed closely by trismus in 25 cases (32.1 %). Additionally, 17 cases (21.8%) presented with both trismus and a burning sensation and in 1 case 1.2%), the clinical symptom was not mentioned. The affected sites displayed blanching in 22 cases (28.2%), while palpable fibrous bands were detected in 18 cases (23.1%). Furthermore, 38 cases (48.7 %) exhibited both blanching and the presence of fibrous bands.

The mean age of the patients was 32.78+10.92 years with age ranging from 18 years to 80 years. The largest proportion of cases fell within the 21-30 years group (41.02%), followed by the 31-40 years group (33.3%), the 41-50 years group (12.8%) and the 20 years and below group (6.4%). The age group with the fewest cases were the ones comprising individual above 50 years and the 20 years and below group (6.4%). There was no significant association observed between the patients' age groups and their respective habits (p = 0.258).

When chewing habits were compared with clinical staging, most cases were concentrated in clinical stage II, with fewer cases distributed across the other stages. There was no statistically significant association between chewing habits and clini-

Table 3: Comparison of fiber color between Group I and Group II

		Group I	Group II	Total	
Fibre color	Green to Greenish yellow	21	4	25	$\chi2$ =22.487 p<0.001, significant
	Yellowish orange	1	13	14	
	Orangish red to red	17	22	39	
Total		39	39	78	

Table 5: Association of fibre thickness with habit in Group II

		Group II				
		Areca nut	Betel quid	Commercial areca nut products	Total	Fisher's exact $\chi2$ =1.219 p=0.689, not significant
Fibre thickness	Thin	2	2	4	8	
	Thick	11	11	9	31	
N		13	13	13	39	

cal staging (p = 0.753), indicating that the distribution of clinical stages did not vary notably with different chewing habits. When the clinical staging and duration of the habit were compared in cases up to 2 years, 2-5 years and more than 5 years of chewing, no significant association was found (p = 0.09). When clinical staging was compared with the frequency of chewing betel quid, areca nut alone and commercial areca nut products, no significant association was observed (p = 0.657).

There was no significant association between histopathological staging and the duration of the habit (p = 0.665). Similarly, the comparison between histopathological staging and the frequency of chewing betel quid (with or without tobacco), areca nut, and commercial areca nut product showed no statistical significance (p= 0.603). Comparison of clinical staging with histopathological grading did not show statistical significance (p= 0.060). (Table 1)

Examination of the PSR stained OSMF sections under a light microscope revealed distinct collagen fiber and fibril arrangement patterns corresponding to each stage of OSMF. In the very early stages, fibrillar collagen was clearly visible with the PSR stain. The early stage demonstrated collagen fibers arranged in separate bundles. In moderately advanced cases, collagen bundles appeared thicker with moderate hyalinization. In advanced stages of OSMF, the collagen was organized into smooth sheets, exhibiting complete hyalinization with only a few remaining separate bundles.

The pattern and arrangement of collagen fibers in different stages of OSMF were more distinctly observed in PSR stained sections compared to conventional hematoxylin and eosin-stained sections. This enhanced clarity is due to the picrosirius red stain's ability to more intensely highlight finer collagen fibers. In comparison of fiber thickness between Group I and Group II OSMF cases, Group I (very mild and mild cases) predominantly displayed thin fibers, while Group II (moderately advanced and advanced cases) primarily exhibited thick fibers. This difference was found to be statistically significant. A significant difference was observed in fiber thickness between Group I and Group II OSMF cases (p < 0.001). Detailed observations are presented in Table 2. A significant difference in fiber color was observed between the Group 1 and Group 2 (p < 0.001), which is presented in Table 3 and Figure 1.

As the disease progressed to more advanced stages, there was a notable shift in the polarization colors of thick fibers, with a significant increase in orangish-red to red hues, and a corresponding decrease in the prevalence of green to greenish-yellow colors. This shift was associated with a marked reduction in the overall number of thin fibers as the disease advanced. However, analysis of fiber thickness (Tables 4 and 5, Fig 2 and 3) and fiber color (Tables 6 and 7) across different chewing habits within Group I and Group II did not reveal statistically significant differences.

Discussion

OSMF is a potentially malignant disorder of the oral cavity, characterized by fibroelastic changes in the lamina propria and epithelial atrophy, leading to trismus and difficulty in eating.18 The pathogenesis of OSMF is multifactorial, but epidemiological studies have consistently identified areca nut consumption, particularly in the form of quid, as the most significant contributing factor.[19,20] The areca nut contains both alkaloid and flavonoid components. Four alkaloids arecoline, arecaidine, guvacine, and guvacoline have been identified of which arecoline is the most potent. Arecoline significantly contributes to the pathogenesis of OSMF by inducing abnormal collagen production. Furthermore, flavonoid components, including tannins and catechins, have been shown to directly affect collagen metabolism.[21]

The distinct histopathological features of OSMF are increased collagen fiber formation in the early stages, which progresses to dense bundles of collagen fibers and varying degrees of hyalinization. As the disease advances, there is also a notable atrophy of the epithelium.[5] Masson's trichrome, Mallory's trichrome, and van Gieson methods are among the most commonly employed connective tissue stains. The van Gieson and

Table 6: Association of fibre color with habit in Group I

		Group I				
		Areca nut	Betel quid	Commercial areca nut products	Total	Fisher's exact $\chi2$ =4.597 p=0.255, not significant
Fibre color	Green to Greenish yellow	9	6	6	21	
	Yellowish orange	3	7	7	17	
	Orangish red to red	1	0	0	1	
N		13	13	13	39	

Table 7: Association of fibre color with habit in Group II

		Group II				
		Areca nut	Betel quid	Commercial areca nut products	Total	Fisher's exact $\chi2$ =1.460 p=0.879, not significant
Fibre color	Green to Greenish yellow	1	1	2	4	
	Orangish red to red	7	7	8	22	
	Yellowish orange	5	5	3	13	
N		13	13	13	39	

PSR stains, in particular, are extensively researched for their ability to stain collagen fibers and exhibit polarizing properties.[16] Sirius Red is an elongated dye molecule that binds to collagen through strong interactions between its acid sulfonic groups and the basic amino acid residues. This binding imparts the property of birefringence, as the dye molecules align parallel to the long axis of each collagen molecule.[15]

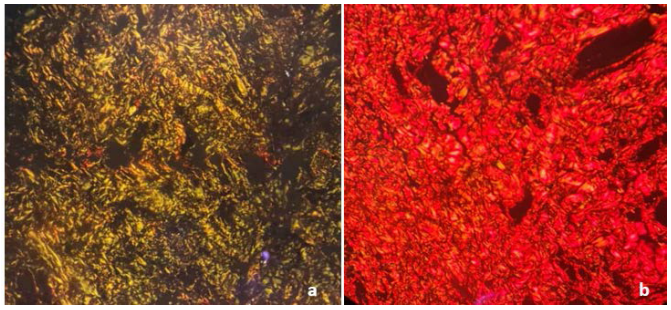

Fig 1: PSR stained sections of Group I showing predominantly green to greenish-yellow birefringence, and Group II displaying mainly orange-red to red birefringence (20x).

The present study shows Group I (very mild and mild cases of OSMF) cases with predominantly thin fibers, whereas Group II (moderately advanced and advanced cases of OSMF) primarily exhibited thick fibers, indicating an increase in thicker fibers as the disease progresses. This observation is corroborated by the fiber color analysis, which revealed that Group I cases predominantly showed green to greenish-yellow hues, while Group II cases exhibited predominantly orangish-red to red colors. These findings are consistent with those reported in previous studies.[17,22] Previous studies have demonstrated that Type I collagen forms thick fibers composed of densely packed fibrils and exhibits intense birefringence with yellow to red coloration when stained with PSR and examined under polarized light microscopy. In contrast, Type III collagen forms thin reticulin fibers made up of loosely arranged fibrils, resulting in weaker birefringence with a green coloration.[16,23,13] The specific colors observed in polarized light microscopy of PSR stained sections may be influenced by factors such as fiber size, alignment, and packing, as well as fiber cross-linking, the interstitial ground substance, and water content.[24]

In India, areca nut is consumed in various forms, from raw

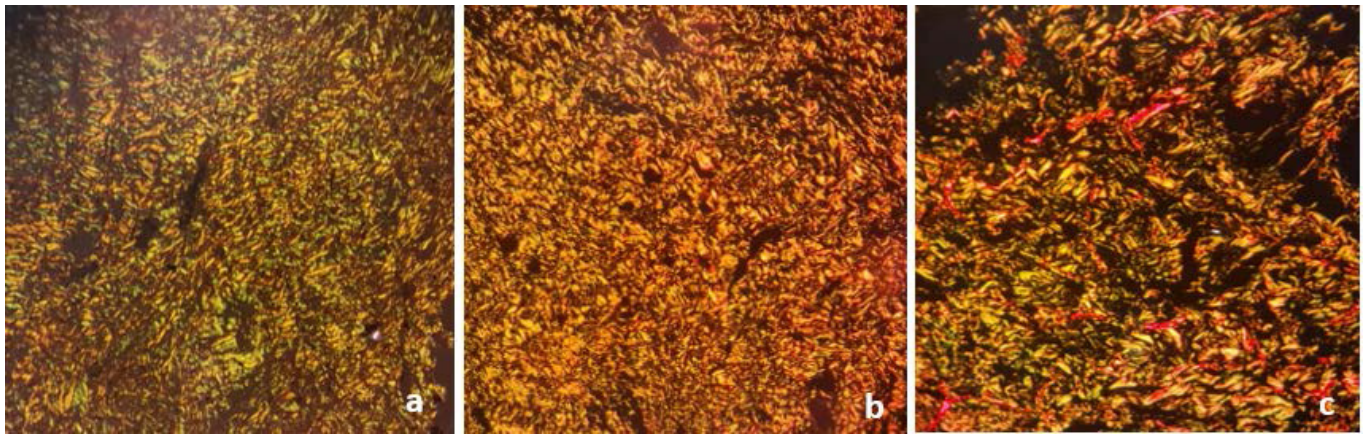

Fig 2: PSR stained sections of Group I cases: Areca nut (a) showing predominantly green to greenish-yellow birefringence, while betel quid (b) and commercial areca nut products (c) exhibit mainly yellowish-orange birefringence (20x).

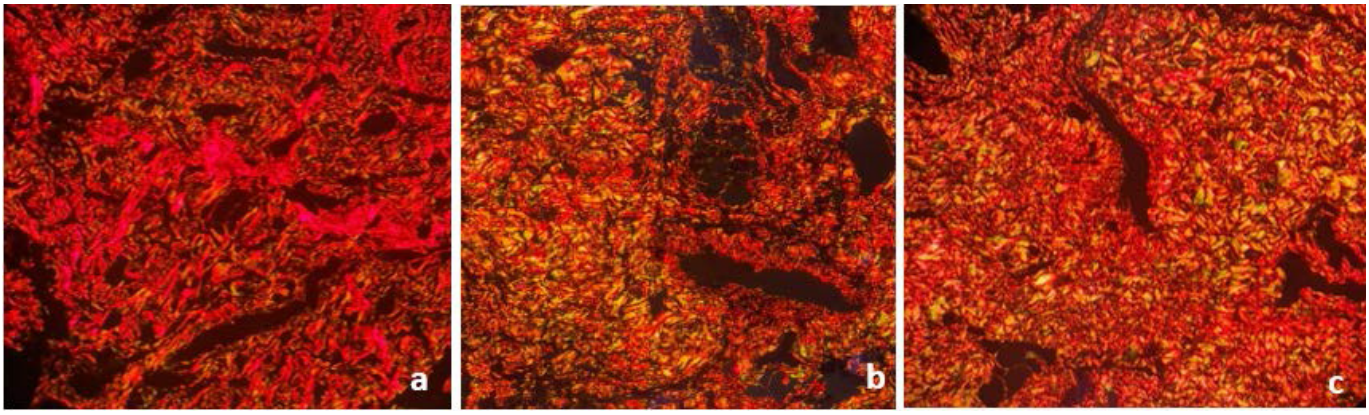

Fig 3: Picrosirius red-stained sections of Group II cases: Areca nut (a), betel quid (b), and commercial areca nut products (c) exhibiting predominantly orange-red to red birefringence (20x).

to commercially processed products. Betel quid typically comprises areca nut, betel leaf, lime, and other potential ingredients such as tobacco and flavorings, though its composition can vary by region. Pan masala, a traditional commercial product, consists of areca nut powder mixed with specified and unspecified additives. When pan masala is combined with tobacco, it is referred to as gutkha.[25] In this study, cases of OSMF associated with areca nut chewing, betel quid (with or without tobacco), and commercial areca products were selected based on Pindborg's histopathological grading system to investigate the impact of different chewing habits on the polarization colors and thickness of collagen fibers. Analysis within Group I and Group II revealed no statistically significant differences in fiber thickness or color across the various chewing habits. This suggests that fiber color and thickness change progressively in accordance with histopathological grading, regardless of the specific chewing habits.

In this study, collagen fiber thickness showed a progressive increase with advancing stages of OSMF. Correspondingly, the polarization colors of the fibers shifted from greenish-yellow in early stages to orangish-red, and eventually to red in more advanced stages. The different chewing habits had no significant impact on either the thickness or the polarization color of the collagen fibers.

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