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Original Research Article

A Polarised Microscopy Investigation using Two Unique Stains for Collagen Fibres in Oral Submucous Fibrosis

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Abstract

Introduction: According to research, the main etiological cause of oral submucous fibrosis is areca nut. Epidemiology studies currently being conducted (OSF). Submucosal fibrosis, which affects the majority of the oral cavity, pharynx, and upper third of the oesophagus, is the disease's hallmark. Detecting collagen fibres in tissues has traditionally relied on stains like Van Gieson and various forms of trichromes, which rely on differential binding by tissue. These stains can distinguish between thick and thin fibres under polarisation microscopy and can be utilised in both light and polarising microscopy. Because of their unique reactivity to most collagen subtypes, picrosirius red dyes are also commonly utilised components.

Material and Methods: The trial group consisted of 100 patients who had been clinically diagnosed with OSMF and agreed to get a biopsy. For patients who were clinically diagnosed with OSMF, a complete case history was taken. Patients were assessed, and relevant information was recorded into Performa, a clinical data collection system. The severity of fibrosis was clinically staged using Lai's categorization, in which the OSMF population was classified into four groups depending on the interincisal distance. Van Gieson Staining and Picrosiriusred staining technique was carried out.

Results: Van Gieson and picrosirius red stains were tested for staining characteristics, overall polarisation colours, and collagen fibre bundle orientation under a polarising microscope. Van Gieson's polarisation was overall reddish, but picrosirius red had a wide variety of birefringence hues, including greenish yellow, yellowish orange, and reddish orange birefringence. A tendency was discovered when collagen fibre distribution in the lamina propria and submucosa were compared. In the lamina propria, reddish orange birefringence was detected in 39% of instances, increasing to 56% in the submucosa. Yellowish orange birefringence was seen in 49 percent of instances in the lamina propria, but only 39 percent of cases in the submucosa. Similarly, greenish yellow birefringence was observed in 14% of instances in the lamina propria, but only 7% in the submucosa. As a result, it's possible that yellowish orange and greenish yellow fibres predominated in the lamina propria, whilst reddish orange fibres predominated in the submucosa. In 90 percent of the Van Gieson stained sections, collagen fibre bundles were arranged parallel to the epithelium. 21 (21%) of the 100 cases had a parallel arrangement of collagen fibre bundles in relation to the epithelium, 75 (75%) had a parallel arrangement with presence of greenish yellow

perpendicular fibres, 4 (4%) had no parallel arrangement, with presence of greenish yellow perpendicular fibres.

Conclusion: Collagen fibres were thicker and more firmly packed in the submucosa than in the lamina propria, indicating that the fibrosis process began there. Because of its stability and ability to stain both thick and thin collagen fibres, Picrosirius red stain outperformed Van Gieson. When stained with Van Gieson, most of the cases showed a parallel arrangement of collagen fibres, but picrosirius red stained sections revealed a majority of parallel type I collagen fibres with perpendicular type III fibres that increased with histopathological grade, suggesting that type III fibres play a role in increased fibrosis**.**

Keywords: fibrosis, histopathological grade, Van Gieson, collagen fibres

Introduction

Oral submucous fibrosis (OSMF) is a severe chronic illness characterised by a juxta epithelial inflammatory reaction followed by a fibro elastic change of the lamina propria with epithelium atrophy, resulting in oral mucosa stiffness, trismus, and inability to chew. According to research, the main etiological cause of oral submucous fibrosis is areca nut. Epidemiology studies currently being conducted (OSF) [1] Submucosal fibrosis, which affects the majority of the oral cavity, pharynx, and upper third of the oesophagus, is the disease's hallmark. To date, areca nut, capsaicin in chillies, micronutrient deficits of iron, zinc, and critical vitamins have been proposed as possible etiological causes. Furthermore, a putative immunological foundation for the condition has been hypothesised, based on the discovery of different autoantibodies and a link to particular HLA antigens. [2] Collagen research has been a mainstay of investigative histology approaches in the investigation of OSMF aetiology. Detecting collagen fibres in tissues has traditionally relied on stains like Van Gieson and various forms of trichromes, which rely on differential binding by tissue components. [3] These stains can distinguish between thick and thin fibres under polarisation microscopy and can be utilised in both light and polarising microscopy. Because of their unique reactivity to most collagen subtypes, picrosirius red dyes are also commonly utilised. [4] Furthermore, combining picrosirius red staining with the polarising microscopy approach improves collagen specificity while simultaneously increasing sensitivity and resolution. [5] Using polarised microscopy, this study was done to qualitatively compare the above two stains in order to analyse the staining capabilities of collagen in OSMF.

Material and Methods

The trial group consisted of 100 patients who had been clinically diagnosed with OSMF and agreed to get a biopsy. All patients gave their informed consent, and the Institute Ethics Committee approved the study.

For patients who were clinically diagnosed with OSMF, a complete case history was taken. Patients were assessed, and relevant information was recorded into Performa, a clinical data collection system. The severity of fibrosis was clinically staged using Lai's categorization, in which the OSMF population was classified into four groups depending on the interincisal distance. [6]

- Group A - more than 35 mm mouth opening

- Group B - a mouth opening of 30 to 35 mm
- Group C - mouth opening between 20 and 30 mm
- Group D - a mouth opening of 20 to 30 mm

After performing the routine hematological investigations, incisional biopsy was done and sent for histopathological evaluation where the specimens were duly processed and stained.

Inclusion and exclusion criteria

The study population included patients with a history of using areca nut in various forms, patients with restricted mouth opening, palpable fibrous bands, and a history of burning sensation when eating spicy foods, and patients with clinically diagnosed and histopathologically confirmed OSMF. Patients having restricted mouth opening due to any cause other than OSMF were excluded from the study, as were patients histopathologically not classified as OSMF.

Van Gieson Staining

To make the stain, dissolve 9 ml of 1 percent aqueous acid fuchsin in 50 ml of saturated aqueous picric acid solution, then add 50 ml of distilled water.

Picrosiriusred staining technique

0.5 g of Sirius Red F3B was dissolved in 500 ml of saturated picric acid solution to make picrosirius red solution. To make acidified water, 5 mL glacial acetic acid was mixed with 1 litre distilled water. Equal parts of solution A and B were mixed together to make Weigerts hematoxylin. Delafield hematoxylin solution and isopropyl alcohol made up solution A, whereas ferric chloride hexahydrate, strong hydrochloric acid, and distilled water made up solution B.

According to Sirsat and Pindborg, the Hematoxylin and Eosin stained sections were studied under light microscopy and cases were classified into very early, early, moderately advanced, and advanced phases based on connective tissue changes. Picrosirius red and Van Gieson staining were done after histological grading, and the stained sections were evaluated using a polarising microscope.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) was used to tabulate and analyse the data (version 16.0).

Results

The study group consisted of 100 people ranging in age from 19 to 65 years old, with an average age of 33.25 7.68 years. With 44 cases, the model age group of 20--30 years had the highest frequency. The majority of the participants had a mouth opening between 20 and 30 mm, putting them in Stage C of clinical staging. The histopathological grading was done according to Pindborg and Sirsat's standards. There was not a single case reported to be in the very early stages; 22 (22%) were diagnosed in the early stages, 71 (71%) in the moderately advanced stage, and 7 (7%) in the advanced stage.

Van Gieson and picrosirius red stains were tested for staining characteristics, overall polarisation colours, and collagen fibre bundle orientation under a polarising microscope at a magnification of 10X. Van Gieson's polarisation was overall reddish, but picrosirius red had a wide variety of birefringence hues, including greenish yellow, yellowish orange, and reddish orange birefringence. Picrosirius red was determined to be a better stain for staining collagen in OSMF than Van Gieson based on staining capabilities and polarisation colours obtained.

The chi square test of association was used to investigate the relationship between clinical staging and histological grading. The chi square test revealed no significant relationship between clinical staging and histological grading (P=0.588), meaning that

histopathological grades were distributed more or less consistently across clinical stages. In 96 percent of the instances, there was muscular invasion.

A tendency was discovered when collagen fibre distribution in the lamina propria and submucosa were compared. In the lamina propria, reddish orange birefringence was detected in 39% of instances, increasing to 56% in the submucosa. Yellowish orange birefringence was seen in 49 percent of instances in the lamina propria, but only 39 percent of cases in the submucosa. Similarly, greenish yellow birefringence was observed in 14% of instances in the lamina propria, but only 7% in the submucosa. As a result, it's possible that yellowish orange and greenish yellow fibres predominated in the lamina propria, whilst reddish orange fibres predominated in the submucosa.

In 90 percent of the Van Gieson stained sections, collagen fibre bundles were arranged parallel to the epithelium. 21 (21%) of the 100 cases had a parallel arrangement of collagen fibre bundles in relation to the epithelium, 75 (75%) had a parallel arrangement with presence of greenish yellow perpendicular fibres, 4 (4%) had no parallel arrangement, with presence of greenish yellow perpendicular fibres.

Discussion

Areca nut is the main etiological cause for oral submucous fibrosis, according to data from current epidemiological research (OSF). It's reasonable to speculate that enhanced collagen synthesis or decreased collagen breakdown is a plausible mechanism in the disease's progression [7]. There are several biochemical pathways involved in the aforementioned activities, and it's possible that normal regulatory systems are down- or up-regulated at various stages of the disease.

The increase of lysyl oxidase in OSMF biopsies supports the potential of copper being a fibrosis mediator, as seen by the high copper concentration in areca nut. South East Asia, East Africa, India, Sri Lanka, Bangladesh, Myanmar, Thailand, Cambodia, Vietnam, the Philippines, and Taiwan, among other areas, have areca nut plantations. It is grown in West Bengal, and North East India region, North and South Kerala, and Coastal Karnataka in India. The betel nut palm thrives in a climate that is consistently wet, with rainfall ranging from 1500 to 5000 mm and temperatures ranging from 15.5 to 38°C.Patient consent declaration.

Using two unique stains and polarisation microscopy, researchers investigated the polarisation colours, orientation, and distribution of collagen fibres in various stages of OSMF. Special stains were used to measure muscle invasion by collagen fibres under light microscopy. The buccal mucosa was the preferred biopsy site, and the surgeon's inability to reach the most posterior portions might cause most stage I cases to progress to stage II, as fibrosis begins at the fauces and moves anteriorly [9]. The degree of trismus may be determined by factors such as the location and extent of fibrosis, regional anatomical variation, muscle tone, neuromuscular coordination, and the physioanatomical integrity of the underlying oral musculature. Clinical staging and histological grading might be thought of as separate entities, and were similar as obtained by Ashalata et al [10] and Rooban et al [11] . Modak et al. found that as histopathological grade progressed, fibrosis and rise in collagen thickness, resulting in clinical trismus [12].

In our study A tendency was discovered when collagen fibre distribution in the lamina propria and submucosa were compared. In the lamina propria, reddish orange birefringence was detected in 39% of instances, increasing to 56% in the submucosa. Yellowish orange birefringence was seen in 49 percent of instances in the lamina propria, but only 39 percent of cases in the submucosa. Similarly, greenish yellow birefringence was observed

in 14% of instances in the lamina propria, but only 7% in the submucosa. According to our findings, the most prevalent colours were yellowish orange and reddish orange, regardless of histological grades, indicating that there was no link between histopathological grading and polarisation colours. Kamath et al. achieved similar results [13].

With both Van Gieson and picrosirius red stains, we found a parallel arrangement of type I collagen fibres in the majority of cases, regardless of histopathological grades. Several additional researchers, including Smitha et al., Parveen et al., and Patel et al., discovered similar parallel collagen fibre orientation to the epithelium [14,15,16].

Conclusion:

Collagen fibres were thicker and more firmly packed in the submucosa than in the lamina propria, indicating that the fibrosis process began there. Because of its stability and ability to stain both thick and thin collagen fibres, Picrosirius red stain outperformed Van Gieson. When stained with Van Gieson, most of the cases showed a parallel arrangement of collagen fibres, but picrosirius red stained sections revealed a majority of parallel type I collagen fibres with perpendicular type III fibres that increased with histopathological grade, suggesting that type III fibres play a role in increased fibrosis.

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