Study of polarization colour in the connective tissue stroma of the odontogenic lesions and to study the role of collagen fibers in the expansion of the lesion by using picrosirius red stain with polarizing microscope

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Abstract

Aim: To evaluate the nature of collagen fibers in odontogenic cysts & tumors by determining the birefringence using picrosirius red stain and polarizing microscope.

Objective:

1. Is to examine the nature of collagen fibers
2. Is to evaluate the orientation of the collagen fibers
3. Is to correlate picrosirius red stain of collagen to biologic behaviour of the lesion.

Materials and Method: Collagen fibers of 23 cases of odontogenic lesion including odontogenic tumors and cysts were studied by staining the sections with picrosirius red stain and examining under polarizing microscope.

Result: 66 percentage of the thin collagen fibers of the radicular cyst and odontogenic keratocyst exhibited green yellow birefringence.100 percent cases of the infected odontogenic keratocyst exhibited orangish yellow birefringence. The odontodenic tumors like Ameloblastoma, Keratocystic odontogenic tumor and Adenomatoid odontogenic tumor exhibits predominantly red colored birefringence. The predominant orange red birefringence exhibited in 33% of OKC, 66% of keratocystic odontogenic tumors 6% of infected OKC along with 66% cases of ameloblastoma and adenomatoid odontogenic tumors exhibited thick and tightly packed collagen fibers. Statistical analysis with one way ANOVA was significant with $p<0.01$. Moreover comparing polarization of collagen fibers with the duration of the lesion also gave statistically significant results.

Conclusion: The observations in the present study with the respect to the color profiles of the collagen fibers in these commonly occurring odontogenic lesions possibly explain the biological behaviour of the lesions. The predominant orange red birefringence in cysts like KCOT, OKC are same as in cases of the odontogenic tumors like ameloblastoma and adenomatoid odontogenic tumor. This may possibly explain the reason for the prognosis of red birefringence showing lesions which suggests that though the epithelium plays an important role in the pathogenesis of the lesions, even stroma is likely to play an equally important role in the pathogenesis and biological behaviour.

Keywords: Collagen fibers, Odontogenic lesions, Polarized microscopy and Picrosirius red.

Introduction

Jaws are the host to various cysts and neoplasm due to the complex tissues involved in the tooth formation. Many of these lesions exhibit aggressive behaviour leading to the bone destruction.(1) Odontogenic lesions comprises of a diverse group of oral lesions which range from hamartomatous proliferation, cysts to benign and malignant lesions.(2) Attempts have been made to understand the biological behaviour of various processes and pathogenesis of the lesion which lead to rendering best health care measures to mankind.(3) The general prevalence of these lesions, their multi-formality effects complexity in understanding pathogenesis and its biological behaviour.(4)

The epithelial-mesenchymal interplay has been known both physiologically and pathologically in relation to odontogenesis. During this interaction in odontogenic apparatus the induction affects both ectodermal and mesodermal tissue which are responsible for such huge variety of form which these odontogenic lesions exhibit, this is the reason why these lesions are most commonly studied, discussed and debated on Pindborg et al. indicated that the odontogenic lesions represent the range of conditions and its features probably depend on the stage of induction towards the tooth formation.(5) The fact that the proliferation of the epithelial cells is an indispensable ingredient for the cyst formation connective tissue should also be regarded as the functional part of the cyst not just mere a structural part.

The connective tissue stroma basically is formed from the fibrous collagenous matrix with proliferating fibroblast having predominantly type I, III and type IV collagen fibers along with elastin, reticulin and oxytalin fibers.(6) Various studies show the inductive changes in connective tissue and that the stroma plays an important role along with epithelium in exhibiting their biological behaviour from its origin to progression. Like the histogenetic differences and clinically aggressive behaviour of OKC in comparison with the other group has led to studies of different cystic aspirates and epithelial linings. Little research is done with respect to

connective tissue wall. Collagen plays a main role in maintaining structural integrity and it determines its tissue function. Therefore many methods were advised to detect, quantify and analyze collagen. To understand the biological behaviour of the lesions various techniques can be employed from histochemistry, immunohistochemistry, electron microscopy and Polymerase chain reaction. There are many stains which selectively stain the stroma like Masson's trichrome, Van geison. However these cannot detect the nature of collagen fibers as they cannot reveal very thin, fine fiber thus not giving the actual status of the collagen in the lesion. Putcher and colleagues observed thin delicate fine fibers with Picrosirius red stain when viewed under polarization microscope.[8,9,10] Collagen molecule being rich in basic amino acids can strongly react with acidic dyes. Picrosirius red dye is an elongated molecule which reacts with the collagen and promotes an enhancement of its normal birefringence. The dye molecules align itself parallel to the long axis of collagen fibers. It is also used because collagen has the inherent nature of birefringence and this stain also stays for long. Picrosirius stain helps to determine their content in specific lesions. It also helps in enhancement in specific collagenous structure. Thick and thin fibers also cause differences in polarization colour.

The lack of understanding of the role of connective tissue in various odontogenic lesions led us to do a study to analyse the nature of collagen fibers using picrosirius red stain with polarization microscope.

Materials and Method

27 cases of the odontogenic lesions were retrieved from the archives. Among these 3 cases were of Radicular cyst, 3 cases were of Odontogenic keratocyst, 3 cases were of Dentigerous cyst, 3 cases were of infected Orthokeratinized odontogenic cyst, 3 cases were of Adenomatiod odontogenic tumor, 3 cases were of Keratocystic odontogenic tumor, 3 cases were of normal mucosa, 3 cases were of Ameloblastoma. The diagnosis of each lesion was confirmed by examining one section stained with Hematoxylin & Eosin using bright field microscopy. The second section was stained with picrosirius red. The picrosirius red stained sections were examined under bright field microscope and polarizing microscope. All the images were clicked under oil immersion and studied for colour birefringence which collagen fibers in different odontogenic lesions exhibit.

Reagents

1. Weigerts iron haematoxylin a(hp-44)-50ml
2. Weigerts iron haematoxylin b(hp-45)—50 ml
3. Sirius red solution (hp 131) 100ml
4. Acetic solution (hp-132)500ml
5. Working solution was prepared by mixing hp-44 and hp-45 in equal volumes before use

2 Sections of 4 micrometer thickness were cut. One section was stained with H & E stain. Another section was stained with picrosirius red solution. Fixed paraffin section were incubated at 47 degree celsius. On warm table deparafinization is done and hydration is done in distilled water. Nuclei were stained with weigerts iron hematoxylin. The slides were then washed with distilled water. The slides were then incubated in 0.1% Sirius red F3B in saturated picric acid solution for 2 hour. This was followed by rinsing with distilled water and staining with Weigerts hematoxylin. Differentiation was done in 1% acid alcohol. Alkalinisation was done in tap water. Dehydration and mounting with dibutyl phathalate xylene (DPX) was done.

To evaluate the birefringence pattern of collagen fibers, all the sections were examined under 20x magnification using Olympus polarized microscope.4 fields of slides were randomly selected. Polarization colour divided into:

- Green(range blue to green)
- Yellow(yellow green –yellow orange)
- Red(orange and red)

In odontogenic cysts predominant colours of collagen fibers in connective tissue in relation to the epithelium were evaluated. For odontogenic tumors the predominant colour around islands was considered.

Data were read independently by three observers to eliminate interobserver bias and were found to be comparable. The results were then tabulated and subjected to statistical analysis using Anova test.

Results

Collagen fibers of the normal mucosa appeared uniformly dark pink (Fig. 1) under bright field microscope and exhibits greenish orange birefringence under polarized light microscopy. 66 percent of the thin collagen fibers of the Radicular cyst (Fig. 2), Dentigerous cyst (Fig. 3) and odontogenic keratocyst (Fig. 4) exhibited orange red birefringence. 100 percent cases of the infected odontogenic keratocyst (Fig 5) exhibited yellow green birefringence. The odontogenic tumors like Ameloblastoma (Fig. 6), Keratocystic odontogenic tumor (Fig. 7) exhibits predominantly yellow green birefringence. The predominant orange red birefringence was seen mainly in radicular cyst and dentigerous cyst which exhibit mature tightly packed collagen fibers. 66% AOT (Fig. 8) cases show orange red birefringence. It also exhibits haphazard arrangement of collagen fibers. Parallel arrangement of collagen fibers were also seen in Dentigerous cyst, OKC, Radicular cyst and Ameloblastoma and KCOT and they exhibited yellow green birefringence. Statistical analysis with one way ANOVA was significant with a $p<0.01$. Moreover comparing polarization of collagen fibers with the duration of the lesion also gave statistically significant results.

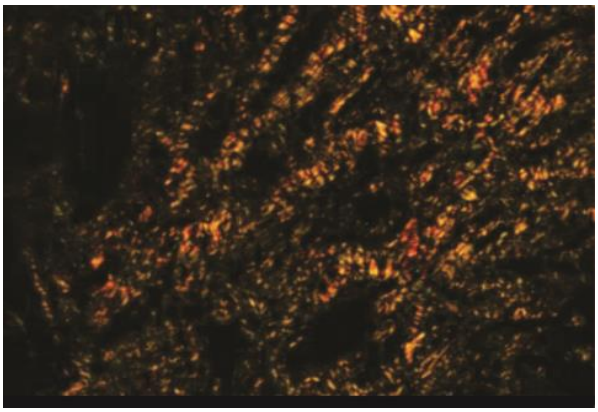

Fig. 1: Normal mucosa exhibiting green birefringence

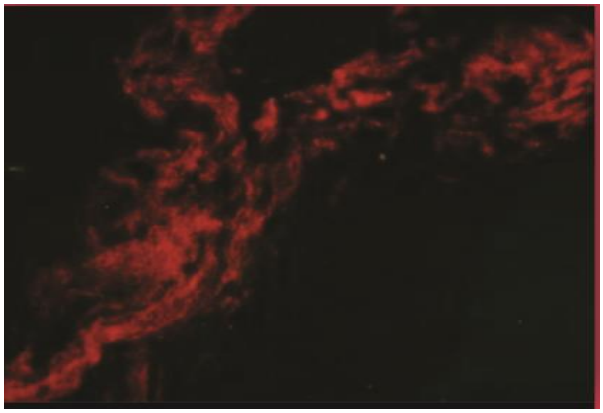

Fig. 2: Radicular cyst exhibiting red birefringence

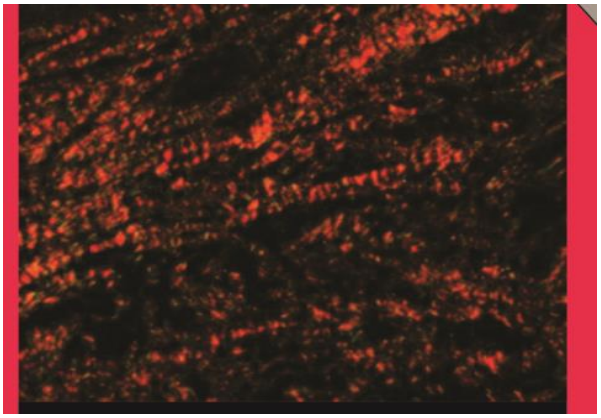

Fig. 3: Dentigerous cyst exhibiting orange red birefringence

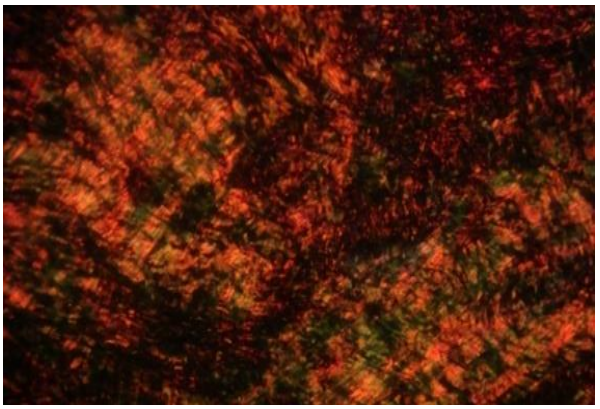

Fig. 4: Odontogenic keratocyst exhibiting orange red birefringence

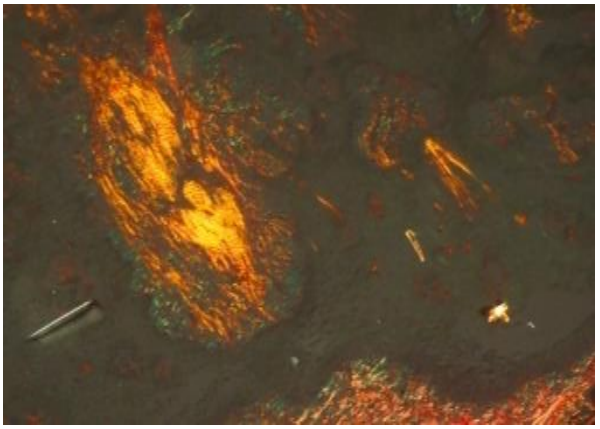

Fig. 5: Infected OKC exhibiting yellow green birefringence

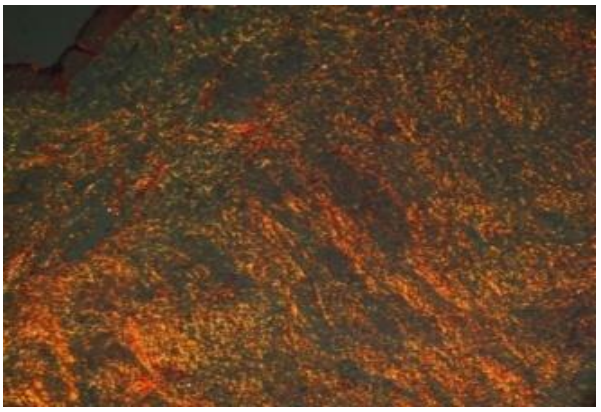

Fig. 6: Ameloblastoma exhibiting yellow green birefringence

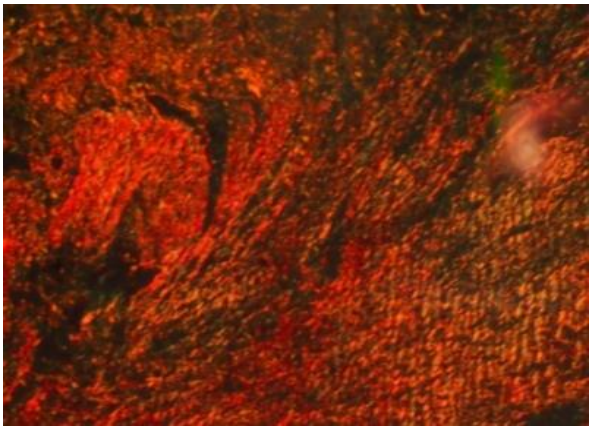

Fig. 7: Keratocystic odontogenic tumor exhibiting orange red birefringence

Discussion

During odontogenesis cell differentiation and morphogenesis is controlled by the epithelial mesenchymal interaction. Thus over a period of time this interplay in tissues participating in odontogenesis lead to the development of the myriad pathologies in the form of hamartomatous lesion, cysts and tumors.[11]

This Epithelial mesenchymal interplay is essential for maintaining homeostatic equilibrium in adults with the stromal cells maintaining control over the cell size, function, response to wounds and other pathologic conditions through modification of the extracellular matrix (ECM).[12] The ECM plays main role in growth regulation, tissue differentiation and organization.[13] The bewildering variety of odontogenic lesions we see is actually due to the complexity which is shown by the induction effects within the odontogenic apparatus. Hirschberg et al. focused on the importance of epithelial mesenchymal interactions in the odontogenic lesions and that the stroma plays a extremely important role.[14]

The collagen is a major organic component which constitutes 34% extracellular protein. Type I and type III collagen are the most predominant types. The collagen exhibits change in the nature and structure of collagen in various pathological condition. Thus the tumor stroma plays an important role in the progression of neoplasm .It act as a physical barrier towards the host immunological reaction. It plays a vital role in supplying essential nutrients to the tumor via blood and helps to remove waste products. The amount of stroma differs from each other.[15]

Various studies on the stromal changes have been discussed which were based on the fact that the collagen is mainly responsible to maintain the functional integrity of tissues. The most common gold standard to study a pathology is histopathology by using H & E stain. Various stains are used to demonstrate collagen fibers and the most commonly used stains are Goldners trichrome stain, Van gieson, Masson trichrome, modified Movats stain, wilder modification of Biechowsky's method, Biechowsky's method wilder modifications.[16,17,18] All stains fail to study the nature and thickness of the collagen fibers, thus sirius red dye was first introduced as a microtechnique. It acted as a substitute for acid fuschin in Van geison staining method. The enhanced birefringence produced by such stains helps in differentiating different types of collagen fibers like procollagen, intermediate collagen, mature collagen and pathologic collagen. It is observed that picrosirius red enhances the normal birefrengence of the collagen fibers.

Picrosirius red stain and polarizing microscope help us to study the individual collagen fibers, size alignment, packaging of fibers, molecular organisation, ground substance and water content.[20,21] It was observed that the collagen displaced different interference colour and intensities of birefringence when studied by picrosirius red because different interstitial collagen displays physical aggregation collagen type I which forms thick fibers composed of closely packed thick fibers and consequently present intense birefringence with yellow to red color.[21,22] Collagen type III forms thin fibers composed of loosely packed thin fibrils and thus they display week birefringence of a green color.[23,24,25,26]

Picrosirius red stain when combined with polarizing microscope exhibits an increased specificity and sensitivity of the methods. It also increases the resolution of the image. The importance of studying epithelial-mesenchymal interaction is that it gives an insight and demonstrates the expansion involved in degradation of bone matrix and cell attachment to ECM components.[27] Thus the study was undertaken to study the staining intensity of the collagen fibers along with the nature of arrangement of fibers by picrosirius red in odontogenic cysts and tumors.

Sharf et al. stated that the colour profile is related to the physical aggregation of collagen that when well packed the fibers exhibit yellowish orange and orangish red and when loosely packed collagen fibers exhibit greenish yellow birefringence.[28] Junqueria et al. suggests that type I is a thick collagen fibers which are loosely arranged and they exhibit week birefringence of green colour. Thick fibers exhibit orangish red birefringence.[29] These findings go in accordance with our study.

Aggarwal and Saxena[27] in their study concluded the similarity between Dentigerous cysts, Odonotogenic keratocyst,and odontogenic tumors which indicates that the lesions have a common histogenesis with broad spectrum of the biological behaviour. Hishberg et al. studied OKC, dentigerous cyst, radicular cyst.[30] Thin fibers exhibit green to green yellow birefringence. It was found that the OKC exhibits both thick and thin fibers showing procollagen, intermediate collagen and pathologic collagen exhibiting greenish yellow birefringence as compared to radicular cyst, dentigerous

cyst. In our study 66% OKC cases showed red birefringence but few cases exhibits green yellow birefringence, which go in accordance with the earlier study suggesting the presence of both thick and thin fibers in odontogenic keratocyst

Like in our case maximum studies show that the inflammatory cyst exhibit predominant green yellow birefringence, the probable explanation is that the collagen fibers are loosely arranged. Cytokines, lymphokines and endotoxins are released by inflammatory cells. Collagenase activity degrades collagen matrix leading to the repair and regeneration of matrix.[30] Teronan et al. macrophages and neutrophils have a common features of elaboration of a tissue collagenase which is capable of causing hydrolytic breakdown of peptide bonds in the helical region of collagen. Collagenolytic activity is least in normal and mildly inflamed tissue and greatest in severely inflammed tissue and hyperplastic tissue, thus the inflammatory cells count affect arrangement of collagen.[31,32,33] Radicular cyst, different pattern of this cyst suggest different biological behaviour. Positive role of inflammation and polarization colour of collagen fibers is seen.

In developmental cyst the longstanding nature of the lesion cause degeneration of the stroma in the form of hyalinization and may explain exhibition of change in collagen fibers. These changes exhibit change in phenotype or thickness of the epithelium. Predominant yellowish orange birefringence in the developmental cyst is seen because it has a well organized collagen fiber network and can also exhibit stromal reaction of odontogenic tumor and thus it explains clinical and histological aggressive nature of these cysts this finding also go in accordance with our study.

Study by Aggarwal and Saxena shows predominance of orange red birefringence in OKC, green yellow colored birefringence's seen in Ameloblastoma and Dentigerous cyst and yellow colored birefringence in Radicular cyst.

Hiershberg and Ahajan et al. in 2017[24] study showed that OKC shows greenish yellow birefringence for both thick thin fibers. If green yellow polarization is seen in radicular cyst, it explains the packing of collagen fibers. Recently it was proposed that the inflammation is necessary to trigger the initiation of fibrosis, tissue destruction, ongoing wound healing responses and eventually fibrosis. This could explain the orange red fibers which were present in Radicular cyst.

When comparing odontogenic tumors with Odontogenic keratocyst on the basis of the colour of the birefringence they exhibit under polarisation microscope it was noted that both of them showed tightly packed collagen fibers. Thus the nature of both the lesion are similar along with its treatment.

It is a well known fact that the dentigerous cyst arise from the expansion of the dental follicle. These follicles exhibits fine delicate collagen fibers which show green birefringence under polarised microscope. As the lesion progresses and matures collagen fibers become tightly packed which brings change in birefringence colour i.e. it changes from green to orange red. This finding also go with our study as new cases of dentigerous cyst exhibited green birefringence and long duration cases exhibited orange birefringence.

Odontogenic keratocyst in our cases showed green yellow birefringence. The reason behind this is that the collagen fibers are loosely packed and are formed from pro-collagen, intermediate collagen and pathologic collagen. These collagen fibers also exhibit correlation with collagenase, prostaglandins and highly oxidative enzymes which degrade collagen and lead to the formation of defective collagen. Presence of the high level of aminopeptidase and high enzyme activity also degrades collagen.[41]

Radicular cyst is an inflammatory cyst which exhibits large amount of inflammation leading to the fibrosis of collagen fibers, these lesions release cytokines and growth factors which promote fibroblastic proliferation. But according to Hishberg few studies disapproves with the fact that inflammation is in direct correlation with fibrosis.[41]

Conclusion

Based on the study it was seen that picrosirius stain act as an important tool in rapid and easy demonstration of collagen fibers and also provide a powerful complement to immunohistochemistry, in situ hybridization and other diagnostic technologies. The observation in the present study with respect to colour profiles of the collagen fibers in the three commonly occurring cysts and few tumors possibly explain the biological behaviour of the lesions. The predominant orange red birefringence in cysts and tumors suggests well organized and tightly packed fibers, this explain the reason for the prognosis.

Therefore it is suggested that though the epithelium plays an important role in the pathogenesis of these lesions even stroma is likely to play an equally important role in the pathogenesis and biological behaviour.

However further investigations on biochemical and molecular studies are required on a larger sample to know the major role of collagen fibers in the pathogenesis of cysts and tumors and also the influence of the mesenchyme in the behaviour of the lesions

Extensive studies with the large samples of such lesions may shield on their biological behaviour especially the aggressive ones thus helping precise treatment planning and patient well being.

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