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ROLE OF CONNECTIVE TISSUE IN AFFECTING THE NATURE OF OVERLYING EPITHELIUM IN VARIOUS ODONTOGENIC CYSTS - A POLARIZING MICROSCOPIC STUDY

Microbiology

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ABSTRACT

Background: Connective tissue often plays an important role in the pathogenesis of odontogenic cysts and there is a necessity to understand the biological behavior of these lesions.

Aims and Objectives: To evaluate the nature of collagen in connective tissue walls of keratocystic odontogenic tumor (KCOT), dentigerous cyst and radicular cyst and compare it with dental follicle.

Materials and methods: A retrospective study was conducted in which 52 histologically diagnosed slides comprising of keratocystic odontogenic tumors (15), dentigerous cysts (15), radicular cysts (15), normal mucosa (2) and dental follicular tissue (5) were stained with picrosirius red and observed under polarized microscope. The interpretations of the color profiles exhibited by the collagen fibers given by Junqueira et al., was considered as standards to interpret the tissue sections. The obtained data were analyzed using SPSS (Statistical Package for the Social Sciences) for Windows (Version 18.0), and Pearson chi-square test was used with statistical significance fixed at 0.05.

Results: Greenish yellow birefringence (GY) was recorded more in keratocystic odontogenic tumor (46.7%) when compared to radicular cyst (13.3%) which was highly significant (P value .088). Reddish orange (RO) was more among the samples of radicular cyst (40%), Greenish yellow birefringence (GY) was predominantly in Dental follicle (60%), and Yellowish orange (YO) was predominantly observed in buccal mucosa (50%).

Conclusion: The appearance of collagen fibres in the keratocystic odontogenic tumor was predominantly different suggesting that connective tissue may be responsible for the biologic behavior of the cysts.

KEYWORDS

Sirius red, collagen, birefringence, polarized light, stroma

INTRODUCTION

Odontogenic cysts and tumors are resultant of epithelial mesenchymal interactions which manifests as multitude of cystic lesions in the jaws.[1] Many studies have suggested that the proliferation of epithelial cell rests is responsible for formation of various odontogenic cysts.[2,3]

Odontogenic cysts can result in many adverse complications such as recurrence along with aggressive growth characteristics.[4]Many studies have been undertaken to understand the recurrence phenomenon and malignant transformation but still it is not very clear.[5,6] Many factors play an active role in the growth and expansion of odontogenic cysts that is in the early and intermediate stages of cystic growth, osmotic pressure play an important role but however, for very large cysts, this role becomes negligible and cell birth in the lining influences the growth.[7]

Stroma is an integral part of the odontogenic cysts which has a functional role as well as providing structural support. Connective tissue often plays an important role in the pathogenesis of odontogenic cysts.[8] Recently, many studies have been conducted to understand the role of connective tissue (stroma) of the odontogenic cysts and its influence on the overlying epithelium.[9,10] The stroma of the odontogenic cysts consists of fibrous collagenous matrix with proliferating fibroblasts, exhibiting predominantly type I and type IV collagen fibers along with oxytalan, elastin and reticulin fibers. Collagen may play a significant role in the expansion of odontogenic cysts.[11,12] It can be inferred from various studies that, odontogenic cysts show a varying degree of inductive changes in the connective tissue and that stroma plays an important role along with the epithelium, in exhibiting their biological behavior from the origin, progression and malignant transformation.

It has been reported that keratocystic odontogenic tumor (KCOT) demonstrated stromal collagen fibers which were different from other odontogenic cysts indicating that stroma of KCOT may play an important role in determining the neoplastic behavior of the lesion through epithelial-mesenchymal interaction.[13] To understand the biological behavior of these lesions; studies on the stromal changes have been employed by various techniques ranging from histochemistry, immuno-histochemistry, electron microscopy and polymerase chain reaction. Various special stains have been employed to study stroma selectively and these include masson's trichome, van geison's and picrosirius red stains.[14]

Picrosirius red stain, an anionic dye that selectively reacts and stains collagen fibers has been of specific importance in differentiating different types of collagen fibers in various oral conditions including odontogenic cysts and odontogenic tumors.[2,15] Collagen, being an anisotropic substance, its packing and nature can be studied in the connective tissue walls of cysts and neoplasms with polarizing microscope. Birefringence is a phenomena exhibited by anisotropic objects which can be selectively visualized by polarized light microscopy.[3] Differences in polarization colors are caused by fibre thickness, as well as by packing of collagen.[16,17]

The birefringence pattern of collagen fibers between various odontogenic cysts such as keratocystic odontogenic tumor (KCOT), radicular cyst, dentigerous cysts and other odontogenic tumors may indicate that these lesions have a common histogenesis with a wide spectrum of biological behavior.[17]

Inflammation in the stroma exhibits different patterns of polarization of colors in various cysts suggesting different biological behavior and a positive role of inflammation.[9] There is a necessity to understand and provide more evidence regarding the nature of collagen fibers in the cyst wall of various odontogenic cysts; hence the present study was undertaken.

AIM AND OBJECTIVES

1. To evaluate the nature of collagen in connective tissue walls of keratocystic odontogenic tumor (KCOT), dentigerous cyst and radicular cyst and compare it dental follicle.
2. To compare the nature of collagen between inflamed and uninflamed cysts and to infer if the underlying connective tissue

has an effect on the biological nature of the overlying epithelium.

MATERIAL AND METHODS

The present study was carried out by retrieving the formalin fixed paraffin embedded tissue blocks of radicular cysts, keratocystic odontogenic tumors, and dentigerous cysts from the Department of Oral Pathology and Microbiology, Jaipur Dental College, Jaipur. The cases coming for the histopathological diagnosis from the Department of Oral and Maxillofacial surgery, Jaipur Dental College Jaipur, during the period of the study were also included. Before the conduction of the study, the protocol was submitted to the ethical review board of Jaipur Dental College, Jaipur and after obtaining ethical clearance, the study was started. A pilot study was performed with 5 samples in each of the odontogenic cysts to test the feasibility and to calculate the sample size. The total material for the study included 52 formalin fixed paraffin embedded tissue blocks. Among these were 15 blocks of histologically diagnosed keratocystic odontogenic tumor, 15 blocks of dentigerous cysts, 15 blocks of radicular cysts and 2 of normal mucosa of buccal mucosa and 5 cases of dental follicular tissue as controls.

METHODOLOGY:

Paraffin embedded tissue blocks were sectioned at 5 um thickness, the sections were floated on to the micro slides and incubated at 47°C on slide warmer for 1 hour for ensuring adhesion of the sections to the slide. Sections were then deparaffinized in xylene and hydrated through decreasing grades of alcohol and taken to water. All the slides were then stained with picrosirius red stain for collagen. The picrosirius red stain often called "Sirius red" is widely used histochemical technique which selectively binds collagen and becomes more specific when observed under polarized light.[18]

EVALUATION:

Labomed Research microscope with polarization lens was used to evaluate picrosirius red stained slides for recording different polarizing colors of collagen in three zones in capsule of all cysts. The zones considered were sub-epithelial zone, intermediate zone and peripheral zone **(Figure 2)**

INFERENCE:

The interpretations of the color profiles exhibited by the collagen fibers given by Junqueira et al.,[19] were considered as standards to interpret the tissue sections in the present study. Under Polarization microscope: Thick, densely packed collagen fibers appear : Yellowish-orange to orange, Thin, loosely packed collagen fibers appear: greenish-yellow. Evaluation of the areas, showing transition from greenish-yellow yellowish-orange to orange birefringence, in same slides under light microscopy was done to see if the color change was associated with any change in the degree of inflammatory cell infiltrate or with any change in thickness or phenotype of the overlying epithelium. Three groups of polarizing colors were made and they were as follows: GY= Greenish yellow, YO= Yellowish orange and RO= Reddish orange. The inflammatory cell density was noted as follows: 0= Absent, 1= Mild, 2= Moderate and 3= Intense. Two observers independently viewed all the slides, to minimize the inter observer variability and the average score of both the observers was considered as final.

Statistical analysis:

Sample size calculation: The sample size was calculated using N master soft ware with the following formula. $\mathbf{n=t^2 \times p(1-p)}$

$\mathbf{m^2}$ ***Description:***

n = required sample size

t = confidence level at 95% (standard value of 1.96)

p = estimated prevalence or distribution

m = margin of error at 5% (standard value of 0.05)

The obtained data were analyzed using SPSS (Statistical Package for the Social Sciences) for Windows (Version 18.0), and descriptive and inferential statistics were used. The Descriptive procedure displayed univariate summary statistics for several variables in a single table and standardized values (z scores) were calculated. Pearson chi-square, likelihood-ratio chi-square, and linear-by-linear association chi-square were used. For 2x2 tables, Fisher's exact test was computed when a table that does not result from missing rows or columns in a larger table has a cell with an expected frequency of less than 5. Yates' corrected chi-square was computed for all other 2x2 tables**.** The statistical significance was fixed at 0.05.

RESULTS

The samples included 15 (28.8%) radicular cysts, 15 (28.8%) keratocystic odontogenic tumor 15(28.8%) dentigerous cysts. The samples of dental follicle 05 (9.6%) and buccal mucosa 2 (3.8%) were taken as controls. **(Table 1).**

Greenish yellow birefringence (GY) was recorded more in keratocystic odontogenic tumor (46.7%) when compared to radicular cyst (13.3%) which was highly significant (P value .088). **(Figure 3)** Yellowish orange (YO) was observed almost in similar proportions in both the cysts. Reddish orange (RO) was more among the samples of radicular cyst 6 (40%) when compared to keratocystic odontogenic tumor (13.3%) which was highly significant (P value - .0088). **(Figure 5)** Greenish yellow birefringence (GY) was predominantly in Dental follicle (60%), and Yellowish orange (YO) was predominantly observed in buccal mucosa 1 (50%). **(Table 2)**

Moderate inflammation was observed predominantly in keratocystic odontogenic tumor (66.7%) when compared to radicular cyst (33.3%) which was statistically significant (P- 0.044). Intense degree of inflammation was recorded maximum in the samples of radicular cyst (53.3%) which was statistically significant (P value- 0.044) **(Table 3).** Based on the chronic inflammatory cell distribution in the sub-epithelial zone, intermediate zone and peripheral zone, various degree of inflammation was recorded in all the odontogenic cysts.

DISCUSSION

Odontogenic cysts and tumors have drawn attention in recent times because of their biological behavior and their neoplastic potential. The stroma or connective tissue is thought to play a significant role in the pathogenesis and biological behavior. Epithelial-mesenchymal interactions in various odontogenic cysts and tumors such as keratocystic odontogenic tumor (KCOT) have been the focus of several studies. It has been demonstrated that transplanted KCOT epithelium in nude mice retained its typical histological appearance only when supported by its own stroma which suggests that the biologic behavior of keratocystic odontogenic tumor is dependent on both, the epithelium and the underlying stroma.[20]

In the present study, the samples of radicular cysts stained with picrosirius red stain and observed under polarizing microscopy, the collagen fibers demonstrated yellowish orange (YO) and reddish orange (RO) predominantly in all the zones. Different color intensities of birefringence were observed in the same tissue section of radicular cyst, indicating a difference in collagen packing in the connective tissue sub-epithelial zone. **(Table 3),** where as the keratocystic odontogenic tumor showed predominantly greenish yellow (GY) and yellowish orange (YO) indicating pro-collagen, intermediate, or pathologic collagen fibers suggestive of loosely packed collagen fibers.

The findings of our study is in agreement with studies conducted by Kulkarni PG et.al[16] who demonstrated that in odontogenic follicles and dentigerous cysts the color birefringence was predominantly orange-red; where as in unicystic ameloblastoma and KCOT showed both orange red and greenish-yellow birefringence; and fibers of multicystic/ solid ameloblastoma showed predominant greenish-yellow birefringence and ameloblastic carcinoma that showed almost complete greenish birefringence. Similarly Singh H et.al [3] et al have reported greenish yellow collagen fibers in multilocular KCOT when compared to unilocular whereas orange red were significantly more in unilocular variety. Our study also corroborates with the findings of the studies by Raj Y et al.[17], Sadik H et al.[21] Kaijkar MS et.al[22], and Mahajan AM et al.[8]

Our findings differs from the findings of Jahagirdhar PB et al[10] who reported the appearance of collagen fibers to be predominantly yellowish orange birefringence among developmental cysts when compared to the yellowish orange and orangish red to red birefringence exhibited by odontogenic tumors suggesting tightly packed fibers.

The stroma may play an important role in the pathogenesis of these lesions influencing changes in the epithelium which may contribute to neoplastic transformation. Since, neoplastic growth requires a functional stroma; the ability of neoplastic cells to induce the formation of such a stroma is of great importance. The aggressive nature of KCOT when compared to other odontogenic cysts may be

due to increase in young and immature collagen fibers in the connective tissue wall.[17] The loose collagen fibers have greenish to greenish yellow birefringence which gives rise to its aggressive characteristics.[23] The results of the present study demonstrate that the composition of the mesenchymal component of keratocystic odontogenic tumor differs from that of dentigerous and radicular cysts. Whether this has any effect on the epithelial lining of the keratocystic odontogenic tumor is yet to be investigated.

The thickness of the collagen fibers may also contribute for the distinctive color birefringence in various odontogenic cysts and tumors. Dayan D et.al[24] reported that most thin fibers of collagens showed green to yellowish-green polarization colors where as thick fibers were reported to have green to greenish-yellow polarization colors, while in all others were found to range from yellowish-orange to red which demonstrated that packing of collagen plays an important role in the pattern of color birefringence of collagen fibers along with thickness. Our findings are consistent with the pioneer study by Hershberg A et al.[15], which was carried out to assess the difference between that the differences in collagen packing in the connective tissue of OKC in comparison with other odontogenic cysts and corroborates with findings of Hershberg A et. al[15] and Vij R et.al[25]

Inflammation also has an impact on the packing of collagen fibers in the connective tissue wall of OKC as reflected by their birefringence colors under polarized light. In the presence of dense inflammation, the percentage of thick fibers with green birefringence decreases, with an increase in thick fibers with red birefringence which appeared more packed.[2]

In the present study, odontogenic cysts showed a mild, moderate to intense inflammatory cells in the connective tissue wall. Although there was an observable amount of inflammation in the sub-epithelial zone, the picrosirius red stain imparted a yellowish orange birefringence to collagen indicating an accumulation of mature closely packed fibers. This change in intensity could be related to mature capsular tissue unlike the embryonic tissue of keratocystic odontogenic tumor and dentigerous cysts. It has been postulated in few of the studies that during maturation of fibers, the proteoglycan content changes, and dehydration occurs, then diameter of collagen fibers increases notably and leads to increase in intensity of birefringence and change in polarization colors.[26]

Our findings are consistent with the findings of these studies by Hershberg A et al[2], Vij R et al[25], Kaijkar MS et al[22], Singh H et al[3], who have reported that shift in color in case KCOT and dentigerous cyst was attributed to the presence of inflammation in the connective tissue. Loosely packed fibers predominantly exhibiting green birefringence had mild inflammation when compared to predominantly green and yellow birefringence in moderate cases and in severely inflamed cases which had well-packed and thick fibers, orange to red birefringence were recorded. Thus, indicating that inflammation influences polarization color and packing of collagen fibers in the connective tissue wall of infected and odontognic cysts and also responsible for change in behavior of neoplastic epithelium of KCOT, whereas in dentigerous it was responsible for changes in epithelial lining.

CONCLUSIONS

The Picrosirius red stain in conjunction with polarizing microscopy may serve as a specific and sensitive tool in characterizing the nature of collagen fibers in wall of various odontogenic cysts. The presence of Greenish Yellow birefringence predominantly among keratocystic odontogenic tumors exhibits loosely packed pathogenic fibers which may be responsible for aggressive characteristics. Further studies with an increased sample size and using various epithelial and mesenchymal markers and antibodies should be carried out to confirm the effect of epithelial–mesenchymal interactions on the nature of epithelium of odontogenic cysts.

TABLES

Table 1: Showing total number and type of samples included in the study

Type of Cysts		Number (N)	Percentage
Inflammatory	Radicular Cyst	15	28.84 %
Developmental	Odontogenic Kerato Cysts(Okc)	15	28.84%
	Dentigerous Cyst	15	28.84%
Controls	Dental Follicle	05	9.61%
	Buccal Mucosa	02	3.84%
Total		52	

Table 2: Distribution Of Colour Birefringence In Subepithelial Zone, Intermediate Zone And Peripheral Zones Of Various Odontogenic Cysts, Dental Follicle And Buccal Mucosa

Group	Sub epithelial Zone			Intermediate Zone			Peripheral Zone		
	GY	YO	RO	GY	YO	RO	GY	YO	RO
Radicular Cysts	2(13.3%)	7(46.6%)	6(40%)	0(0)	9(60%)	6(40%)	1(6.6%)	10(66.6%)	4(26.6%)
Odontogenic Keratocysts	9(60%)	6(40%)	0(0)	7(46.6%)	7(46.6%)	1(6.6%)	2(13.3%)	13(86.6%)	0(0)
Dentigerous Cysts	5(33.3%)	10(66.6%)	0(0)	0(0)	12(80%)	3(20%)	0(0)	14(93.3%)	1(6.6%)
Dental Follicle	4(80%)	1(20%)	0(0)	4(80%)	1(20%)	0(0)	3(60%)	2(40%)	0(0)
Buccal Mucosa	1(50%)	1(50%)	0(0)	1(50%)	1(50%)	0(0)	2(100%)	0(0)	0(0)
Statistics	Pearson Chi square value - 23.230 df- 8 p value-.003			Pearson Chi square value - 22.215 df- 8 p value-.005			Pearson Chi square value - 33.681 df- 8 p value-.000		
GY- Greenish Yellow YO- Yellowish Orange RO- Reddish Orange									

Table 3: Distribution Of Chronic Inflammatory Cells In Sub Epithelial Zone, Intermediate Zone And Peripheral Zone Of Various Odontogenic Cysts, Dental Follicle And Buccal Mucosa

Group	Sub epithelial Zone				Intermediate Zone				Peripheral Zone			
	0	1	2	3	0	1	2	3	0	1	2	3
Radicular Cysts	0(0)	0(0)	7(46.6%	8(53.3%)	0(0)	3(20%)	9(60%)	3(20%)	3(20%)	8(53.3%)	3(20%)	1(6.6%)
Odontogenic Keratocysts	3(20%)	2(13.3%)	10(66.6%)	0(0)	4(26.6%)	4(26.6%)	7(46.6%)	0(0)	8(53.3%)	6(40%	1(6.6%)	0(0)
Dentigerous Cysts	1(6.6%)	7(46.6%)	6(40%)	1(6.6%)	6(40%)	5(33.3%)	3(20%)	1(6.6%)	2(13.3%)	6(40%)	7(46.6%)	0(0)
Dental Follicle	3(60%)	2(40%)	0(0)	0(0)	1(20%)	3(60%)	1(6.6%)	0(0)	2(40%)	2(40%)	1(10%)	0(0)
Buccal Mucosa	1(50%)	1(50%)	0(0)	0(0)	0(0)	2(100%)	0(0)	0(0)	2(100%)	0(0)	0(0)	0(0)
Statistics	Pearson Chi square value- 35.934 df - 12 p value-.000				Pearson Chi square value- 20.433 df - 12 p value-.053				Pearson Chi square value- 21.115 df -12 p value-.049			
1. Nil 1- Mild 2- Moderate 3- Intense												

Figure Legends

Figure 1 showing condensor and polarizer

Figure 2 showing Labomed research microscope

Figure 3 Photomicrograph showing collagen fibers in KCOT with greenish yellow birefringence under polarizing microscopy in 10X

Figure 4 Photomicrograph showing collagen fibers in dentegerous cyst showing yellowish orange birefringence under polarizing microscopy in 10X

Figure 5 Photomicrograph showing collagen fibers in radicular cyst showing reddish orange birefringence under polarizing microscopy in 10X

Figure 1 showing condensor and polarizer

Figure 2 showing Labomed research microscope

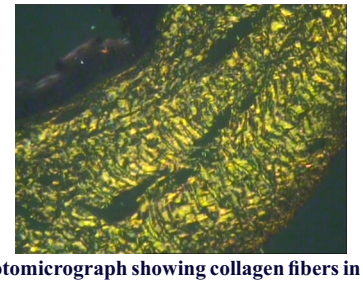

Figure 3 Photomicrograph showing collagen fibers in KCOT with greenish yellow birefringence under polarizing microscopy in 10X

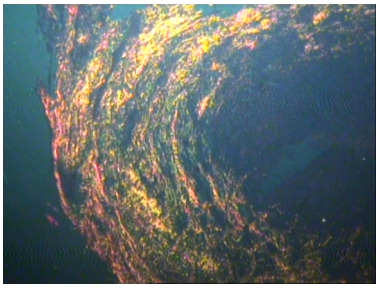

Figure 4 Photomicrograph showing collagen fibers in dentegerous cyst showing yellowish orange birefringence under polarizing microscopy in 10X

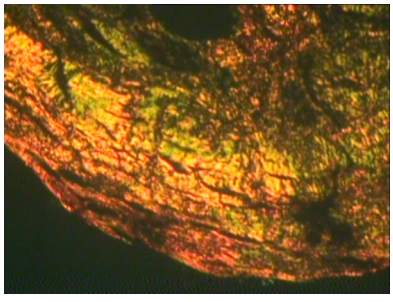

Figure 5 Photomicrograph showing collagen fibers in radicular cyst showing reddish orange birefringence under polarizing microscopy in 10X

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