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Comparison of Picrosirius Red Staining of Collagen Fibers of Keratocystic Odontogenic Tumor with Odontogenic Cysts

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Abstract: *Intricate relationship between epithelium and stromal tissue is believed to play a significant role in pathogenesis of odontogenic lesions. Role of epithelium in its pathogenesis is well established and at the same time role of mesenchyme cannot be underestimated. This study was undertaken to investigate, compare and correlate different pattern of distribution of collagen fibres in Keratocystic odontogenic tumor with other odontogenic cysts using picrosirius red stain under polarizing microscopy in order to delineate its exact role in biological behavior of these lesions.*

The colour of collagen fibres in the fibrous tissue walls of 30 Keratocystic odontogenic tumor,10 cases each of dentigerous cysts, periapical cysts and orthokeratinised odontogenic cysts were studied by staining with picrosirius red and examining under polarizing microscope. Results were statistically analyzed using SPSS software. Thick collagen fibres of Keratocystic odontogenic tumor and periapical cyst showed predominant colour of greenish yellow birefringence, whereas that of dentigerous cyst and orthokeratinized odontogenic cyst was found to be orange red. Birefringence of thin collagen fibres didn't show any difference between the groups. Hence we concluded that quality of collagen fibers was different in each group which accounts for the difference in biological behavior of these lesions.

Keywords*: Birefringence, Collagen, Keratocystic odontogenic tumor, Picrosirius red*

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I. Introduction

Odontogenesis is a highly co-ordinated and complex process which relies upon cell to cell interactions and cell to matrix interactions that result in the initiation and generation of tooth[1]. Following odontogenesis, the odontogenic epithelium degenerates and forms odontogenic remnants. These remnants may get triggered by unknown mechanisms to form pathological lesions. Lesions of odontogenic origin comprise of a heterogeneous group ranging from hamartomatous proliferations, cysts to benign and malignant tumors[2]. Odontogenic cysts are expansile lesions with favorable prognosis whereas tumors are destructive lesions with variable prognosis[3]. The most commonly encountered cysts in the oral cavity are radicular cyst (52.3%),dentigerous cyst (18.1%), and odontogenic keratocyst (OKC) (11.6%)[4] OKC is notorious for its high recurrence rate which ranges from 10 to 62% [5]. Also, the growth of this cyst is more unremitting; Scharfetter in 1989 stated that the invasive growth of keratocysts is likely to be the result of active growth of the connective tissue wall. In human keratocyst collagenase degrades type I and II collagens at almost equal rates, but at the same time, no significant degradation of type III collagen occurs, implying that destruction of this connective tissue is associated with the growth of keratocysts[6].The proposed histogenetic differences and the clinically more aggressive behavior of odontogenic keratocysts compared with other cyst types has brought about studies aimed at characterizing possible differences between their fluid aspirates and epithelial linings. Little research has been done with respect to their connective tissue walls[7].

Recent evidence of the intrinsic growth potential within the epithelial lining of the OKC led to its reclassification as a tumor by the WHO in 2005 [8].The term Odontogenic keratocyst (OKC) was first used by Philipsen in 1956 to designate an odontogenic cyst that demonstrated keratinization of its lining epithelium. The new designation of OKC that is Keratocystic odontogenic tumor (KCOT) will better convey its neoplastic nature. KCOT is characterized by parakeratinized epithelial lining which differs histologically and behaviourally from the orthokeratinized odontogenic cyst-OOC.[5] KCOT is a clinicopathologically distinct form of odontogenic cyst, known for its pathognomonic microscopic features, aggressiveness and high recurrence rate[9].This is the most aggressive and recurrent of all the odontogenic cysts and shows characteristics resemblance both as a cyst and a tumor [10].

The biologic behavior of cysts and tumors is dependent not only on the epithelium present but also on the stroma supporting the epithelium. Since collagen forms the major component of the stroma; the study of collagen fibers could be of diagnostic significance[11]. In pathological conditions, collagen can show variations in the way the individual fibrils are organized into fibers and in terms of the diameter and the cross sectional profile. Hence, study of the organization of collagen fibers may be helpful in understanding the behavior of the lesion [12]. Collagen has natural birefringence, which is attributed to the arrangement of its fibers. This property is enhanced by Picrosirius Red dye [13].

Picrosirius red staining in combination with polarization microscopy has been used to study the individual collagen fibers and to determine their content in the specific tissue. Sirius red is an elongated dye molecule which reacts with collagen and promotes an enhancement of its normal birefringence as the dye molecules align parallelly along the long axis of each collagen molecule. This method can serve as a procedure to differentiate pro-collagens, intermediate and pathological collagen fibers which are not tightly packed, from normal packed fibers. [10,14,15]

The present study was under taken to evaluate the nature of collagen fibers in KCOT and to compare it with other odontogenic cysts (Periapical cyst, Dentigerous cyst and Orthokertatinized odontogenic cyst) .

II. Materials And Methods

This cross sectional comparative study was conducted at Government Dental College, Kottayam. 60 formalin fixed and paraffin embedded archival specimens were retrieved from the Department of Oral pathology and microbiology, following obtainment of information regarding age, gender and diagnosis. The study group comprised of 2 groups, with each group consisting of 30 histopathologically confirmed cases of Keratocystic odontogenic tumour(KCOT) and other odontogenic cysts. Group I consisted of 30 diagnosed cases of KCOT. Group II, consisted of 30 diagnosed cases of odontogenic cysts which were divided into three groups consisting 10 cases each of dentigerous cysts, periapical cysts and orthokertinized odontogenic cysts.Only those cases histopathologically diagnosed as non-inflamed Keratocystic odontogenic tumor and noninflamed odontogenic cysts were included in the study. (except periapical cyst.)

30 blocks of KCOT and 30 blocks of odontogenic cysts were sectioned and then stained with picrosirius red stain and Weigert's hematoxylin and viewed under both bright field and polarized microscope. Collagen fibers appear uniformly dark Pink under Bright field microscope and in birefringence colors under Polarized light microscopy. Polarization colors were determined separately for thin and thick fibers. With the application of image analyzer soft ware(**Nis Elements BR 4 version**) fiber thickness measuring 0.8um or less was grouped under thin fibers and thickness between 1.4um and 2.4um were grouped under thick fibers. Fiber thickness was determined on the images captured, using image analyzing software (**Nis Elements BR4 version**) after proper calibration. In each section, three separate high power fields with at least 10 fibers of each size were examined. The connective tissue in these slides showed polarization colors varying from greenish-yellow to yellow-orange to orange red. The data obtained from image analysis software was exported to Microsoft excel master chart for further interpretation and statistical analysis. The color of the collagen fibers was noted by two independent observers to eliminate the subjective bias.

III. Results

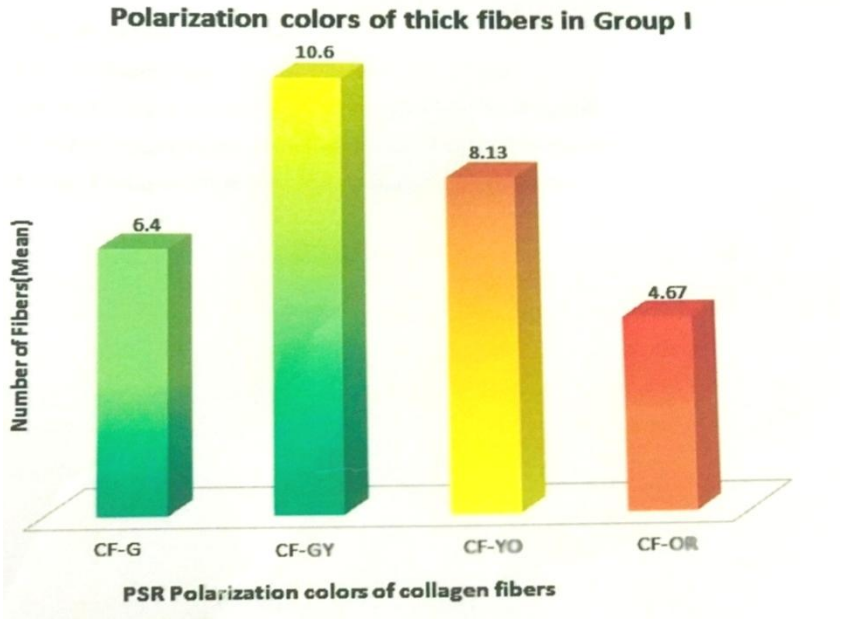
The birefringence of colors observed under polarizing microscope (Nikon Eclipse Ni-U) was noted as follows:
1. Green 2. Green-yellow 3. Yellow-orange 4. Orange-red

Table 1: Distribution of polarization colors of thick fibers in Group I

Color observed	CF-G	CF-GY	CF-YO	CF-OR
No of Fibers	6.47± 2.825	10.60± 3.820	8.13± 2.751	4.67± 2.279

Group I : KCOT-Keratocystic odontogenic tumor
CF-G : Collagen fibers showing green birefringence
CF-GY : Collagen fibers showing green-yellow birefringence
CF-YO : Collagen fibers showing yellow-orange birefringence
CF-OR : Collagen fibers showing orange-red birefringence.

The present study found that picrosirius red polarization of collagen fibers in group 1 (KCOT-" Fig 1") was predominantly green yellow (10.6± 3.82), followed by yellow-orange (8.13± 2.751), green (6.47± 2.825) and orange red (4.67± 2.279) as indicated in Table 1 Graph 1.Both green and green yellow fibers together constituted 57 of total number of fibers.



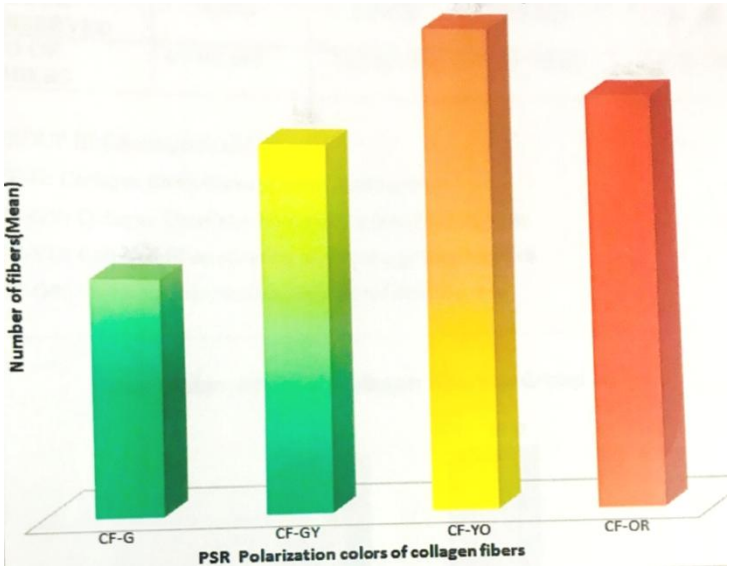
Graph 1 : Distribution of polarization colors of thick fibers in Group I

Table II : Distribution of polarization colors of thick fibers in Group II

Color observed	CF-G	CF-GY	CF-YO	CF-OR
No of fibers	4.63± 3.429	7.30± 3.446	9.67± 3.325	8.33± 4.596

Group II : Odontogenic cysts
CF-G : Collagen fibers showing green birefringence
CF-GY : Collagen fibers showing green-yellow birefringence
CF-YO : Collagen fibers showing yellow-orange birefringence
CF-OR : Collagen fibers showing orange-red birefringence.

In group II (Odontogenic cysts) the predominant picrosirius red polarization of collagen fibers was found to be yellow orange (9.67± 3.325) which was then followed by orange red (8.33 ± 4.596), green yellow (7.30± 3.446) and green(4.63± 3.429). as indicated in Table II Graph II.That means 60% of total number of fibers showed birefringent colors ranging from yellow to orange-red.



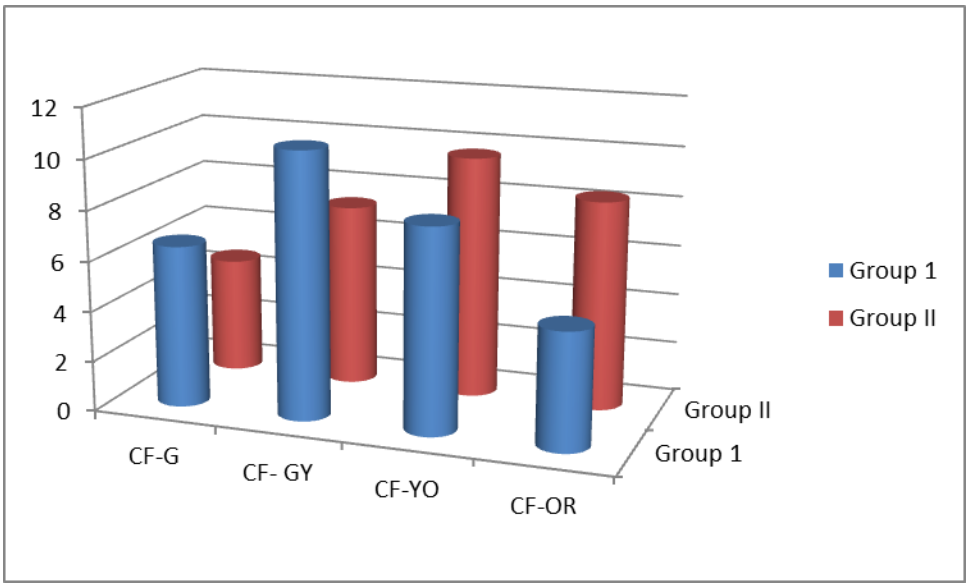
Graph II : Distribution of polarization colors of thick fibers in Group II

Table III : Comparison of polarization colors of thick collagen fibers among the two groups.

Fiber color	Group 1	Group II	t value	P value
CF-G	6.47+ 2.825	4.63+/-3.429	2.26	0.028*
CF- GY	10.60+ 3.820	7.30+/-3.446	3.51	0.001*
CF-YO	8.13+ 2.751	9.67+/-3.325	-1.94	0.057
CF-OR	4.67+ 2.279	8.33+/-4.596	-3.91	0.000*

Group I Keratocystic odontogenic tumor, Group II Other odontogenic cysts.

*p value is <0.05. The differences in mean are statistically significant between the groups. Unpaired t test is used for statistical analysis.



Group I Keratocystic odontogenic tumor, Group II Other odontogenic cysts.

Graph III : Comparison of polarization colors of thick collagen fibers among the two groups

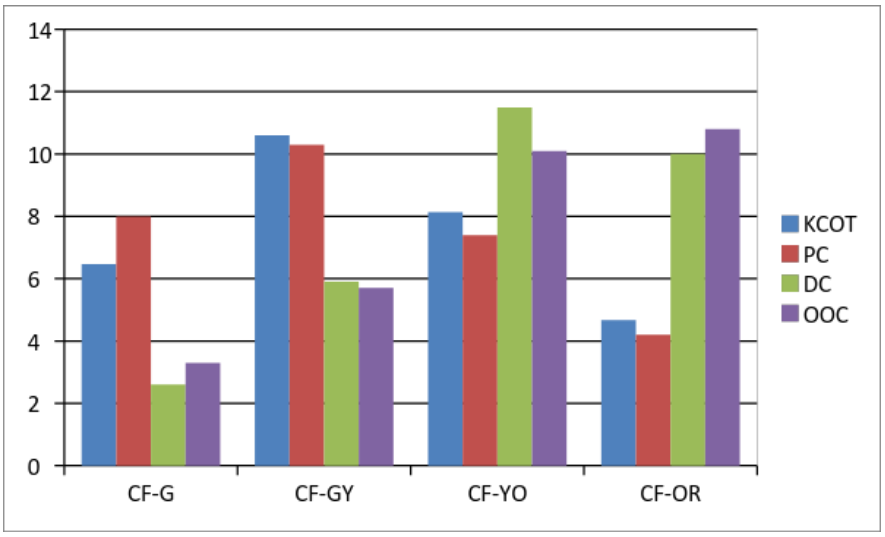
Comparison of all polarization colors of the collagen fibers between 2 groups, indicated a predominant birefringence of green yellow followed by yellow orange, orange red and green with a significant P value less than 0.05 as shown in Table III and Graph III.

Table IV: Comparison of polarization colors of thick collagen fibers among 4 subgroups.

Group	CF-G	CF-GY	CG-YO	CF-OR
KCOT	6.47+2.82	10.6+3.82	8.13+2.75	4.67+2.27
PC	8+ 1.88	10.3+2.40	7.4+2.27	4.2+1.93
DC	2.6+0.84	5.90+3.41	11.5+3.02	10+4.73
OOC	3.3+2.83	5.70+2.40	10.10+3.41	10.8+3.61
F value	10.40	8.814	5.030	17.08
P value	0.000*	0.000*	0.004*	0.000*

PC –Periapical cyst, DC- Dentigerous cyst, OOC- Orthokeratinised odontogenic cyst

Recorded birefringent colors were statistically analyzed by using one way ANOVA test in SPSS version 14. Level of significance was established with P value. The P value less than 0.05 is considered as significant. Comparison of polarization colors of 4 subgroups shows high level of significance and is given in Table IV Graph IV.



Graph IV : Comparison of polarization colors in 4 subgroups

6.47± 2.825 and 10.6± 3.820 thick fibers of KCOT showed green and greenish yellow birefringence (Table 1 and Graph1) which is significantly higher than that of dentigerous cyst. (CF G: 2.6± 0.84 ; CF GY: 5.9± 3.414 ; "Fig 2",Table IV) and orthokeratinized odontogenic cyst(CF G: 3.3± 2.830 : CF GY : 5.70± 2.406 ; " Fig 3 ",Table IV) .Periapical cyst shows 8± l.886 and 10.3 ± 2.406 ("Fig" 4) thick fibers of green and green yellow birefringence respectively. The value is significantly higher with respect to dentigerous cyst (DC), Orthokeratinized odontogenic cyst and KCOT. After analysis of long wavelength birefringent colors like yellow and orange red, the findings obtained were as follows, KCOT showed 8.13± 2.751 yellow orange and 4.67 ±2.279 orange red thick fibers which was significantly lower value than that of dentigerous cyst and orthokeratinized odontogenic cyst.

11.5± 3.028 thick fibers' show yellow orange and 10± 4.738 thick fibers show orange red polarization colors which is significantly a higher value in dentigerous cyst. In OOC 10.10+/- 3.414 thick yellow orange fibers and 10.8+/- 3.615 thick orange red fibers were observed. Hence thick fibers having colors ranging from yellow to red was higher in DC than OOC. In the case of periapical cyst 7.40± 2.27 and 4.2 ±1.932 thick fibers were observed as showing yellow orange and orange red birefringence.

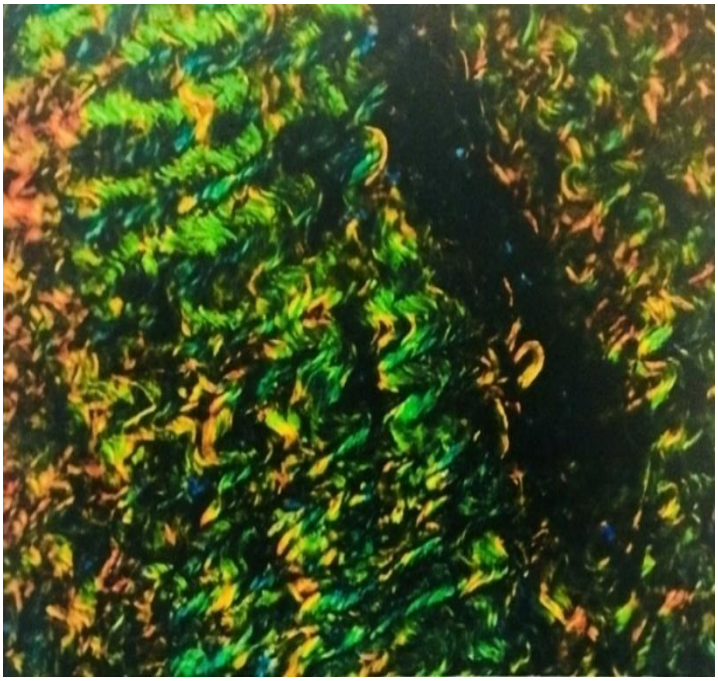


Fig 1: PSR stained keratocystic odontogenic tumor showing predominantly greenish yellow birefringence.

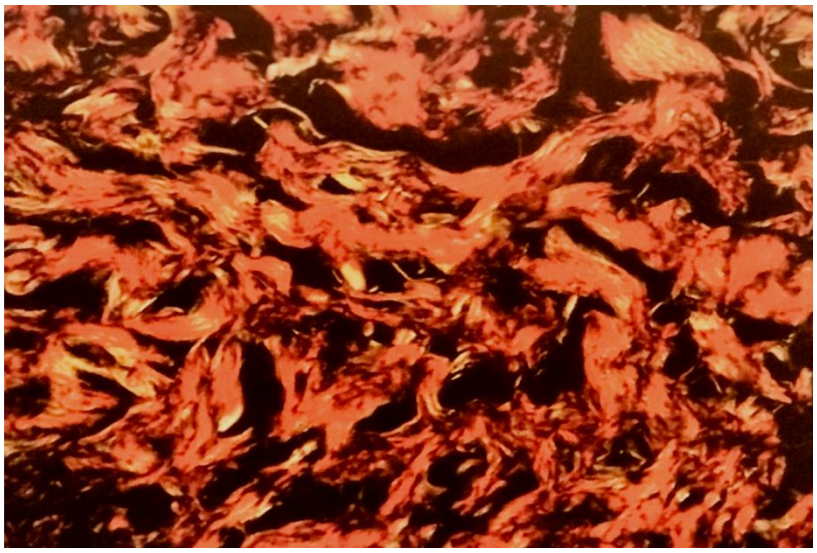


Fig 2 : PSR stained section of Dentigerous cyst showing orange red birefringence

Fig 3 : PSR stained section of Orthokeratinized odontogenic cyst showing predominantly yellowish Orange birefringence.

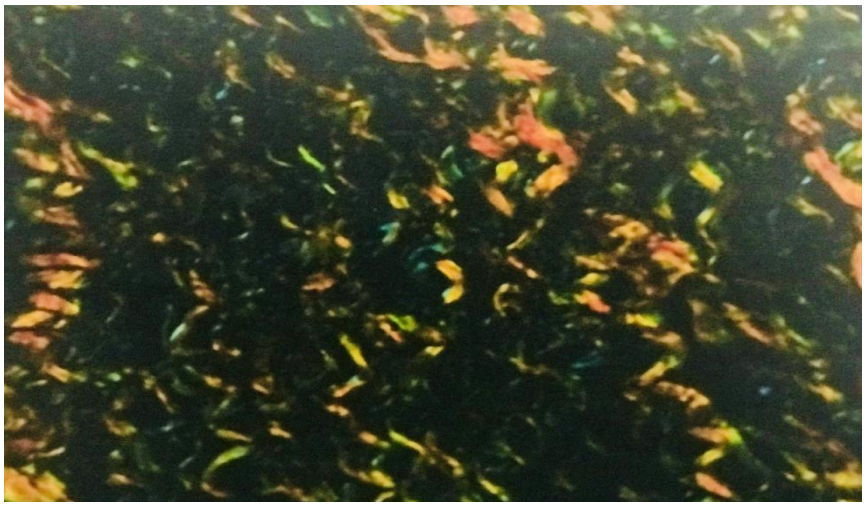


Fig 4 : PSR stained section of Periapical cyst showing greenish yellow birefringence

IV. Discussion

Most of the studies have focused on the evaluation of the proliferative activity in the epithelial component of the odontogenic lesions but the role of mesenchyme still remains unrevealed[16].It was Vedtofte etal [17] who showed the importance of stromal component from a study on the transplanted keratocyst epithelium in nude mice which led the authors to believe that the differentiation of the cystic epithelium is not independent of the stroma and suggested that the stromal component has a role to play in the biological behavior by establishing ectomesenchymal interaction. A recent study showed that CD105 (endoglin) is strongly expressed in microvessels of KCOT compared to dentigerous cysts and normal oral mucosa. This suggests that angiogenesis may be associated with the locally aggressive biological behavior of KCOT[18]. All these findings suggest that the stroma of KCOT could be regarded not just as a structural support of the cyst wall, but as playing a part in the neoplastic behavior of the cyst. The possibility of primary defect of OKC in the mesenchymal capsule rather than in epithelial cells was mooted by Browne. [19]

Stromal changes in these odontogenic lesions can be depicted by picrosirius red stain and this stain imparts birefringence to collagen fibers specifically. According to Constantine and Mowry, PSR stain does not impart birefringence to structures that lack collagen[20]. Imparted colors of collagen fibers range from shades of green, yellow to orange red in various lesions . The color exhibited by these fibers depends on fiber size, alignment and packing of fibers, molecular organization, ground substances and water content. Normally, green to greenish yellow corresponds poorly packed fibers whereas orange- red represents well packed fibers[21]. The use of polarization microscopy to identify collagen in picrosirius stained materials considerably increases the specificity and resolution of the collagen fibers, as even very thin fibrils of collagen undetectable in normal microscopy become visible as a source of light against a dark background. [21,22]

The present study is an attempt to reassess the aggressive nature of individual lesions based on histopathology by studying the polarization color of the collagen fibers in the connective tissue compartment. In the pathological setting, collagen can show variations in the way by which individual fibrils are organized into fibers and in terms of diameter and cross-sectional profile. [23] Collagen Types I, II and III show different colors and intensities of birefringence in the same tissue section as these exhibit different interstitial collagens and display distinct pattern of physical aggregation. Thus, collagen type I forms thick fibers composed of closely packed thick fibrils and consequently presents an intense birefringence with yellow to red color. [21,24]] Collagen type II, shows a variable color according to the tissue and the species. Its color and morphology however always permitted its clear distinction from collagens type I and type III. Collagen type III (reticular fibers) forms thin fibers composed of loosely packed thin fibrils and thus display a weak birefringence of green color.[25] Type I and Type III collagen are the most predominant [26,27,28] types .

Ruchieka vij etal,[29] in his study on collagen in the connective tissue walls of odontogenic cysts employing the Picrosirius staining technique, demonstrated that differences exist in the birefringent color of collagen in the walls of inflamed versus non inflamed cysts. For this reason, areas of inflammation were excluded from examination in our study with exception of periapical cysts.

In the present study, most cases of Keratocystic odontogenic tumor (n 20) were found to be occurring in the fourth decade followed by the third and the second decade respectively. Also, in our present study we found a male predominance with a male to female ratio of 1.5:1 which was in par with the findings of Brannon.[30] Clinically, Keratocystic odontogenic tumor displays a tendency to grow in an anteroposterior direction within the medullary cavity without causing obvious bony expansion and may present with pain, swelling, discharge, paresthesia and appears as a well-defined radiolucency with corticated margins. The diagnosis of Keratocystic odontogenic tumor is based on correlation of clinical, radiologic and characteristic histologic features. The cyst capsule is composed of collagen fiber consisting of both thin and thick fibers arranged in bundles.[19]

Increased expression of collagenase, gelatinases (MMP-1 and MMP-9) (matrix metalloproteinases) and also notable expression of MMP-2 and MMP.8 were found in the tissue extracts of KCOT in both latent and activated forms[31].These tissue collagenases are capable of hydrolyzing collagen and resulting in the alteration of the collagen content of the connective tissue walls of Keratocystic odontogenic tumor.[32] Hirshberg A et al [33] and Zhang J-Y etal [1] in their studies stated that those fibers with a predominant green-yellow birefringence reflected on the nature of collagen bundles (thin bundles).

In attribution to the above findings, the present study was carried out with the aim of evaluating the nature of the collagen fibers in Group I and Group II and to correlate the nature of collagen fiber bundles with their biological behavior. In a few studies the biological behavior of these lesions reflected on the nature of the collagen bundles within their connective tissue wall .[34,35,15] In the present study, cases of KCOT demonstrated a predominant color of green yellow (56.66%," Fig" 1) birefringence within the connective tissue wall which is significantly a higher value than other odontogenic cysts. These findings were consistent with the studies conducted by Hirshberg A etal [33] and Zhang JY etal [1]

These findings may be due to the result of increased degradation of type I and II collagens at almost equal rates, but at the same time, no significant degradation of type III (immature)collagen in the connective tissue wall of KCOT.[6]This would make KCOT to expand at a more rapid rate and may contribute to its aggressive clinical behavior and a high recurrence rate. Studies on KCOT suggests that the collagen found in these lesions is loosely packed and might be composed of procollagens, intermediates, or other pathologic (not tightly packed) collagens. This is in accord with the study by Hirshberg A et al [33] Hirshberg A also reported that staining of collagen fibers in keratocyst was similar to that of odontogenic neoplasms.

Junqueira etal.[36] stated that under pathological conditions birefringence shows a different pattern in comparison with collagen in normal tissue and proved that type I collagen were thick, strongly birefringent red fibers while type III collagen appeared as thin weakly birefringent green fibers.The greenish-yellow birefringence imparted juxta epithelially in KCOT and dentigerous cyst in the present study can be attributed to the young and immature fine type III collagen(dental follicular tissue)[37]. Similar polarizing color profiles were noted in dental follicle in the study of Ruchieka etal [29] Area of collagenolysis appeared as weakly birefringent. This confirms the findings of Philipsen etal who demonstrated ultrastructurally the presence of collagenolysis in the juxta epithelial region of the OKC and is responsible for the ready separation of OKC epithelium from its supporting wall.

In our study, green to greenish yellow color of collagen fibers in periapical cysts suggests that the collagen found in the lesions which is loosely packed might be composed of immature or pathological rather than the normal tightly packed fibers seen in dentigerous cysts and orthokeratinized odontogenic cysts. This could be explained by the fact that the periapical cyst is of inflammatory origin and the inflammatory cells could affect the packing of collagen fibers. The explanation is made in the light of Hirshberg etal studies on collagen pattern in various odontogenic cysts.[38] Teronan etal, held the view that macrophages and neutrophils elaborate tissue collagenase, which is capable of causing hydrolysis of collagen.Thus inflammatory cells could affect the arrangement and packing of collagen. [31]Inflammation in periapical cyst can lead to an alteration in its capsular connective tissue from embryonic tissue to a mature connective tissue. Inflammation has an impact on the packing of collagen fibers in the connective tissue wall of KCOT as reflected by increase in thick fibers with red birefringence and decrease of thick fibers with green birefringence. [38]When we execute this data to our findings, periapical cyst shows more number of greenish yellow fibers which is a conflicting result. This pattern of birefringence may be partly attributed to a possible difference in the intensity of inflammation. Furthermore, the shift in color has also been related to tissue section thickness in a study by Junqueira etal[24] whereas, the study by Hirshberg et al showed that inflammation had a direct influence on the color of the fibers.

In the present study a predominance of yellowish-red color was observed in orthokeratinized odontogenic cyst and dentigerous cyst which originates from well tightly packed fibres. The major polarization color of OOC and DC is yellowish orange and orange red which differs significantly from KCOT, suggesting that the collagen found in these lesions is densely packed and might be composed of native collagen rather than loosely packed fibers seen in KCOT. Owing to different polarization colors the composition of the mesenchymal component of KCOT appeared to be different from that of OOC. It has been demonstrated that the epithelial lining of OOC has low proliferative and self renewal potential compared with that of KCOT, which appeared to reflect the contrasting differences in the biological behavior of these two lesions [39,40]. It is interesting to speculate that differences in collagen fibers' within these two cysts may also be related to their behavioral differences. KCOT is one of the most aggressive odontogenic cyst owing to its relatively high recurrence rate and its tendency to invade adjacent tissue.

In this study, polarization colors of thin fibers were similar in all the two groups and the data found to be insignificant. Thin collagen fibers exhibit green to greenish yellow polarizing colors, with picrosirius red stain. Significant differences in polarization color were demonstrated in the subepithelial zones of KCOT and OOC, suggesting that different collagen fibers may exist in the two lesions and their role in pathogenesis requires further attention. Thus the nature of collagen fibers as studied by the Picrosirius red polarization method may be useful as a diagnostic tool to differentiate between two lesions and may be applied to other pathologic conditions to predict their nature in terms of biologic behavior and prognosis.

V. Conclusion

The present study indicated predominant thick greenish yellow collagen fibers in Keratocystic odontogenic tumor when compared to other lesions. The reason for this has been attributed to dissolution of collagen in Keratocystic odontogenic tumor by liberation of increased amounts of MMP-1, MMP-2, MMP-8, MMP-9 and collagenase targeting loosely packed, denatured collagens in this lesions. Abnormally packed immature collagen is more susceptible to degradation. This feature would make KCOT expand at a more rapid rate and may contribute to its aggressive nature. Greenish yellow birefeingence in periapical cyst is due to the presence of inflammation which end up in increased collagenase activity. Polarization color is depended on several factors like fiber thickness, orientation, organization and its spatial distribution. All account for different

birefringence. To add evidence to the pattern of distribution of collagen and its biological behavior by merely looking up the polarization colors is infact trivial. Further studies are required to correlate other molecular determinants for aggressiveness using picrosirius red polarization of collagen fibers with higher sample sizes to substantiate the real contribution of collagen fibers.

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