www.jmscr.igmpublication.org
Impact Factor 5.84
Index Copernicus Value: 71.58
ISSN (e)-2347-176x ISSN (p) 2455-0450
crossref DOI: https://dx.doi.org/10.18535/jmscr/v5i9.159

IGM Publication
Journal Of Medical Science And Clinical Research
An Official Publication Of IGM Publication

Role of Thickness of Collagen Fibers in Odontogenic Keratocyst and Dentigerous Cyst– A Picrosirius Red Polarized Microscopic Study

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Abstract
Background: *Collagen fibers have a role in the pathogenesis of odontogenic cysts. The precise role of epithelium has been described in detail but still the role of connective tissue in pathogenesis of odontogenic cysts is not yet well established.*
Aims and Objectives: *To assess the influence of collagen in the expansion of odontogenic cysts OKC and DC and also to correlate the thickness of collagen fibers by picrosirius red stained section.*
Materials and Methods: *We carried out our study in 20 histopathologically confirmed cases of odontogenic keratocyst and dentigerous cyst, retrieved from the archives of the department. Histochemical staining of the sections was carried out with picrosirius red stain and then observed under polarized microscope. Results were analysed statistically.*
Results: *Odontogenic keratocysts when compared with dentigerous cysts showed more greenish yellow birefringence of thick fibers and orange red birefringence of the thin fibers.*
Conclusion: *Difference in birefringence of collagen fibers by picrosirius red polarization method can be a helpful diagnostic means to distinguish odontogenic keratocyst and dentigerous cyst. This may help us in predicting the biologic behavior and prognosis of odontogenic cysts.*
Key Words: *Collagen fibers, Dentigerous cyst, Odontogenic keratocyst, Picrosirius stain.*

Introduction

Epithelial mesenchymal interactions are essential for odontogenesis as well as pathologies like odontogenic cysts and tumors. Basically these pathologies arise from the remnants of the odontogenic apparatus, that when triggered proliferate.[1]

Cysta of maxillofacial regions are broadly categorised as odontogenic and non odontogenic cysts. Odontogenic cysts are further classified based on their origin and clinicopathological criteria. Studies have shown that connective tissue stroma play an important role in the pathogenesis and behaviour of odontogenic cysts and tumors

via epithelial mesenchymal interactions. Altered epithelium brings about changes in collagen, with fibers of different quality and organization.[2]

The structure of collagen is described as consisting of 3 polypeptide chains arranged in a triple helix arrangement with glycine, proline and 4-hydroxyproline repeats. Collagen metabolism is influenced by matrix metalloproteinases (MMPs) like collagenases and gelatinases.[2]

Among the odontogenic cysts, odontogenic keratocyst (OKC) is supposed to be aggressive one. It is thought to arise from dental lamina or of basal cells of oral epithelium. Its aggressive behaviour has prompted to rename it as kerato-cystic odontogenic tumour. The pathogenesis of OKC is mediated by many factors like MMPs released by osteoclasts which dissolute bone matrix, collagenase enzyme mediated degradation of connective tissue in cyst wall and by interstitial collagenases and gelatinases found in cystic fluid.[3]

Dentigerous Cyst (DC) is thought to arise from reduced enamel epithelium. Its cystic expansion is by osmotic pressure and bone destruction through MMP's present in cyst wall.[4] Connective tissue mainly consists of collagen. Hence many authors have concentrated on studying collagen fibers in odontogenic cysts. Special stains used to differentiate collagen from other tissue components are Van-Geison, Mallory and Gomori stains. But these special stains were unable to differentiate collagen fibers in stroma from structures like reticulin fibers and basal membranes that contain collagen. Hence by making use of the birefringence property of collagen, picrosirius polarization method was developed to selectively identify collagen fibers. The methodology employed consists of initial picrosirius red staining followed by viewing under polarizing microscopy, which further increases collagen specificity and also resolution of collagen fibers. The basis is fiber thickness and arrangement of collagen molecules. The acid sulfonic groups of sirius red dye binds to basic groups of collagen in such a way that their long axes are parallel resulting in enhanced birefringence. Thus the very thin collagen fibers, usually undetectable in normal microscopy, happen to be visible by this method.[3-5]

Generally polarization color of fibers depends on thickness, thin normal collagen fibers showing green to greenish yellow and thick fibers showing yellowish orange to red. Collagen is classified into various types. Type 1 collagen is made up of closely packed thick fibers (2-10 mm) and it shows an intense birefringence of yellow to red color. Whereas type II collagen is made up of very thin fibrils and does not form fibers, and show a weak birefringence of varying colors. However type III collagen (reticular fiber) is made of loosely dispersed thin fibers (0.5-1.5mm) and show a weak birefringence of greenish color. Hence the study of collagen fibers is established to have a diagnostic significance in various skin lesions, odontogenic cysts and tumors.[4, 5]

Many studies have shown that collagen fibers are arranged differently in odontogenic cysts, being mature, closely packed in dentigerous cysts and immature, loosely packed in OKC, thus showing greater expansion potential in OKC.[6]

We carried out our study to assess the influence of collagen in the expansion of odontogenic cysts i,e OKC and DC and also to correlate the thickness of collagen fibers by picrosirius red stained section.

Materials and Methods

Our retrospective study included 40 formalin fixed paraffin embedded tissue blocks constituting 20 histopathologically confirmed cases of OKC and DC each, retrieved from the archives of the department.

Procedure: The sections were stained with Harris Hematoxylin for 8 minutes and then the slides were washed for 10 minutes in running tap water. Then they were stained with picrosirius red stain for one hour and washed in two changes of acidified water. Then dehydrated in three changes of 100% Ethanol and cleared in Xylene and mounted in resinous medium. Thus stained sections were observed under polarized microscope.

The picrosirius red stained sections showing collagen fibers within the connective tissue wall of OKC and DC were captured using 3 chip CCD camera (Proview, Cybernatics) with 40x objective. Fiber thickness was determined on the image captured, using PROEXPRESS 8 IMAGE ANALYSIS SOFTWARE. All the measurements were made in microns (Fig 1 and 2). Polarization colors were determined separately for thin fibers (0.8µm or less) and for thick fibers (1.62.4µm). In each sections, five separate high power fields in which at least 10 fibers of each size range were examined in three zones of the connective tissue i.e, Sub epithelial zone, Intermediate zone and Peripheral zone. The data from the image analysis was transferred to Microsoft excel sheet for further interpretations and statistical analysis (ANOVA) was done.

Results

Statistical Analysis: Standard ANOVA analysis has been done and it showed significant quantitative differences of polarization colors of the fibers between the two cysts. A p-value of <0.005 was considered statistically significant.

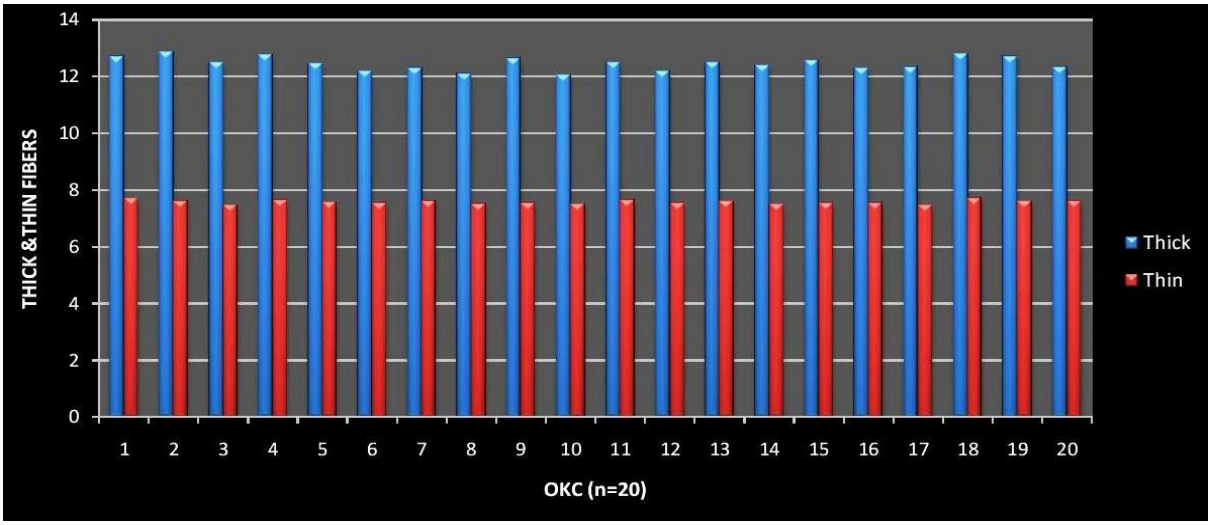
We found that the mean value of thick fibers showing greenish yellow birefringence in OKC and DC were 4.15±0.11 SD and 3.63±0.19 SD respectively, with a significant p value of 0.01. However, the mean value of thin fibers showing orange red birefringence in OKC and DC were 2.52±0.24 SD and 1.94±0.04 SD respectively, with a significant p value of 0.03 (Table 1, Graph 1).

Table 1: Comparison of Thick and Thin fibers in different Odontogenic cysts

	OKC	DC	P Value
Thick Fibers	4.1551	3.6373	<0.01*
Thin fibers	2.5280	1.9440	<0.03*

*p-value – statistically significant

Graph 1: Thick and thin Fibers of OKC



In subepithelial zone, the mean value of thick fibers showing greenish yellow birefringence in OKC and DC were 3.97±0.11SD and 3.66±0.24SD respectively with a significant p value of 0.018 (Table 2).

Table 2: Comparison of Thick fibers of OKC and DC in subepithelial zone

	Cysts (n=60)	Mean	SD	P Value
Subepithelial Zone	OKC(n=20)	3.9795	.11033	0.018*
	DC(n=20)	3.6685	.24355	

*p-value – statistically significant

Whereas in the Intermediate zone, the mean value of thick fibers showing greenish yellow birefringence in OKC and DC were 4.46±0.17 SD and 3.76±0.13 SD respectively with a significant p value of 0.000 (Table 3).

Table 3: Comparison of Thick fibers of OKC and DC in Intermediate zone

	Cysts (n=60)	Mean	SD	P Value
Intermediate Zone	OKC(n=20)	4.4660	.17653	0.000*
	DC(n=20)	3.7665	.13720	

*p-value – statistically significant

Whereas in the Peripheral zone, the mean value of Thick fibers showing orange red birefringence in OKC and DC were 4.02±0.06 SD and 3.47±0.20 SD respectively with a significant p value of 0.012 (Table 4).

Table 4: Comparison of Thick fibers of OKC and DC in Peripheral zone

	Cysts (n=60)	Mean	SD	P Value
Peripheral Zone	OKC(n=20)	4.0200	.06018	0.012*
	DC(n=20)	3.4770	.20131	

*p-value – statistically significant

We found that in subepithelial zone, the mean value of thin fibers showing orange red birefringence in OKC and DC were 2.11±0.37 SD and 1.97±0.83 SD respectively with a significant p value of 0.015 (Table 5).

Table 5: Comparison of Thin fibers of OKC and DC in Subepithelial zone

	Cysts (n=60)	Mean	SD	P Value
Subepithelial Zone	OKC(n=20)	2.1160	.03705	0.015*
	DC(n=20)	1.9780	.08358	

*p-value – statistically significant

In the Intermediate zone, the mean value of Thin fibers showing orange red birefringence in OKC and DC were 2.85±0.20 SD and 2.06±0.29 SD respectively with a significant p value of 0.000 (Table 6).

Table 6: Comparison of Thin fibers of OKC and DC in Intermediate zone

	Cysts (n=60)	Mean	SD	P Value
Intermediate Zone	OKC(n=20)	2.8560	.02037	0.000*
	DC(n=20)	2.0690	.02936	

*p-value – statistically significant

Whereas in the Peripheral zone, the mean value of Thin fibers showing orange red birefringence in OKC and DC were 2.61±0.48 SD and 1.78±0.22 SD respectively with a significant p value of 0.012 (Table 7).

Table 7: Comparison of Thin fibers of OKC and DC in Peripheral zone

	Cysts (n=60)	Mean	SD	P Value
Peripheral Zone	OKC(n=20)	2.6120	.04873	0.012*
	DC(n=20)	1.7850	.02212	

*p-value – statistically significant

Discussion

In the past cystic expansion in the odontogenic cysts were thought totally due to changes in the osmotic pressure. In recent times, many authors are concentrating on the role of epithelial-mesenchymal interactions in odontogenesis and pathologies like odontogenic cysys and tumors. These interactions are thought to modify extracellular matrix (ECM), with collagen being the most important organic component of ECM (approximately 34%).[5, 6] Hence studies have been carried out in predicting the role of collagen, if any in the pathogenesis of odontogenic cysts like OKC and DC. Collagen when stained with Picrosirius red stain and viewed under polarized microscope display diverse interference colors and birefringence intensities, varying from green to greenish yellow to orange red. Hence, Picrosirius–

Polarization method is an exceptionally simple, specific, sensitive, consistent and cost effective method for observing collagen fibers.[7, 8]

Experiments by Vedtofte et al in nude mice showed that the behavior of OKC depends on both the epithelium as well as underlying stroma.[9] In DC, connective tissue behaves like a reactive tissue and initiates an expansile growth.[10, 11]

We carried out a retrospective study on 40 cases previously diagnosed as OKC and DC (20 each) to assess the thickness of collagen fibers which are responsible for expansion of the cysts either directly or indirectly.

Our study results revealed that the number of thick fibers in OKC showing green to greenish yellow color was significantly higher than the thick fibers of DC. Our findings are in accordance with Hirshberg et al[6] and Zhang et al[1]. According to them collagen is loosely packed and hence stroma of OKC might play a vital role in determining the behavior of the lesion through epithelial mesenchymal interaction. Whereas Yukti et al[12] found more greenish yellow birefringence of thick fibers and stated that young and immature collagen might have a say in aggressive nature of cyst.

Our findings are in contrast to Agarwal et al[13] who found that the stroma of OKC, DC and other odontogenic tumors revealed mainly orange red fibers, suggesting that they belong to same group i.e. are developmental in origin and have closely packed fibers.

We found that thin fibers that reveal orange red birefringence were considerably more in OKC than DC. Our findings were in accordance with that of Hirshberg et al.[6] We assessed polarization colors of connective tissue in three zones in each section, subepithelial zone, intermediate zone and peripheral zone. Furthermore comparison of the values obtained was made between the fibers in all three zones and in cysts in each zone.

When polarization colors of collagen fibers in three different cysts in individual zone were assessed, we observed thick collagen fibers showing greenish yellow birefringence predominated in OKC in the intermediate zone when compared to DC. Our results were in accordance with findings obtained from Rucheika et al.[7]

Schrafeter et al[3] suggested that the active growth of stroma might be responsible for the invasive growth of keratocyst. According to them enzymes present in the walls of OKC like beta-napthylamidase and leucine aminopeptidase might result in collagenolysis which leads to separation of epithelium from its underlying connective tissue, thus attributing to its high recurrence rate.[14]

Singh et al[11] showed that collagen fibers were arranged parallel to epithelial surface in most cases of OKC (80%), followed by DC (40%) showed a less percentage of it. According to them this parallel and loose arrangement of collagen fibers might facilitate the separation of epithelial lining from the connective tissue wall. Hence the quality and arrangement of collagen fibers considerably influence the tensile strength of connective tissue and subsequently its capacity to support tissues and organs. In DC, non parallel fibers induce an expansile growth related with fluid accumulation.

In our study the presence of greenish yellow thick fibers in OKC as established by polarized microscopy indicated that the collagen fibers were poorly packed and were composed of procoll-agens, intermediate and pathologic collagen. This proves that stroma of keratocysts might probably play an important role in its aggressive behavior. Whereas the less prevalence of greenish yellow fibers in DC might indicate that collagen fiber bundles are more closely packed and are perhaps not under the influence of intense collagenolytic activity in contrast to OKC.[8]

Our study suggests that OKC is more aggressive lesion than DC by means of identifying abnormally packed collagen fibers in it. Thus the nature of collagen fibers as studied by picrosirius red polarization method may be useful diagnostic tool to differentiate between these cysts and may also help to predict their nature in term of biologic behavior and prognosis. We suggest future such studies with a larger study sample and with more

parameters like crosslinking, alignment and packing of fibers, epithelial mesenchymal interactions at a molecular level, in order to accurately evaluate the role of collagen fibers to differentiate odontogenic cysts.

Conclusion

We found that the thick fibers in OKC were more greenish yellow when compared to DC suggesting that they are loosely packed and more susceptible to degeneration by collagenases, than seen in DC. This arrangement of collagen fibers might contribute to its aggressive nature.

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