Original article

Comparative Evaluation of Polarization Colors of Collagen Fibers in Odontogenic Keratocyst and Ameloblastoma Using Picrosirius Red Stain

(Histochemical Study)

Hawaida M.O Bin Hussain[1], Azzam A. Sultan[2].

[1]Assistant lecturer, [2]Proffessor; Department of Oral Medicine, Oral Pathology, Diagnosis and Radiology, Faculty of Dentistry, University of Benghazi.

ABSTRACT

Aims: studying the quality and quantity of collagen fibers may help to delineate their influence on the aggressiveness and growth potential of the OKC and ameloblastoma. The aims of this study were to evaluate and compare the polarization colors, orientation, and organization of collagen fibers in the connective tissues of the odontogenic keratocyst (OKC), and ameloblastoma (AB) using picrosirius red (PSR) stain under a polarizing microscope with correlation to their biologic behavior.

Materials and method: polarization colors, orientation, and organization of collagen fibers in pre-diagnosed cases of OKC and AB, twenty each, and two control cases of dentigerous cyst (DC) were histochemically analyzed using PSR stain and a polarized light microscope.

Results: comparing the collagen fibers in the two lesions, there was no significant difference with respect to the polarization colors, orientation, and organization.

Conclusion: PSR staining and polarizing microscopy is a powerful tool to appraise the nature and arrangement of collagen fibers in odontogenic lesions. The connective tissue of OKC showed a predominance of green-yellow birefringence, and loosely packed fibers, this can be correlated to the aggressive nature of this lesion.

Keywords: *polarization colors, picrosirius red stain, polarizing microscope.*

Corresponding author:
Hawaida M.O Bin Hussain: Department of Oral Medicine, Oral Pathology, Diagnosis and Radiology, Faculty of Dentistry, University of Benghazi.
E-mail: houida.ramly@gmail.com

INTRODUCTION

Odontogenic tumors and odontogenic cysts are common lesions of the oral cavity, the epithelium, and mesenchymal tissues of the tooth-forming organ may give rise to a variety of odontogenic tumors, also odontogenic cysts are known to be derived from the epithelial rests lying in jawbones.[1]

The odontogenic keratocyst (OKC) was described in 1956 by Philipsen as a developmental odontogenic cyst with distinctive histopathologic features[2]. Ameloblastoma is a benign epithelial odontogenic tumor, it was introduced by Cusack in 1827.[3]

The role of connective tissue of odontogenic lesions regarding their growth potential, aggressiveness, and recurrence has been unfairly overlooked for a long time. Recent studies have underscored the fact that, the epithelium and connective tissue are nearly equally involved in determining and predicting the clinical course of an odontogenic lesion, this holds true as the reciprocal interaction between odontogenic epithelium and mesenchymal tissue occurs at the early stages of odontogenesis, this is also applied to pathologic processes which lead to the development of various odontogenic tumors and cysts.[4-6] Collagens, as a major constituent of connective tissue capsule in the wall of these lesions, can be accurately evaluated using picrosirius red stain and polarizing microscope.[7]

The PSR stain (F3BA) is an anionic dye-containing six sulfonate groups that chemically reacts with basic amino acids-rich collagens, this

acid-base interaction allows each SR to bind to a collagen molecule alongside its axis; subsequently, the natural phenomenon of birefringence is enhanced when exposed to polarized light.[8] Collagen fibers demonstrate green, yellow, and red colors according to the amount of the light absorbed, in which collagens stand out against a black background, the color difference is largely dependent on packing, physical aggregation, and thickness of the fibers.[8,9] This study was conducted in an attempt to evaluate and compare the polarization colors of collagen fiber bundles, and their pattern of orientation and organization in the connective tissues of OKCs and ameloblastoma with correlation to their biologic behavior.

MATERIALS AND METHODS

Sample selection

After requesting and obtaining the ethical approval from the Scientific Research Ethics Committee, University of Benghazi, Faculty of Dentistry to conduct this study, pre-diagnosed cases of OKC and AB; in addition to control cases of DC, were retrieved from the archives of the Department of Oral Medicine, Oral Pathology, Diagnosis and Radiology, Faculty of Dentistry, University of Benghazi, between 1996 and 2019. Cases with well-preserved histopathologic diagnostic criteria were included; whereas, inflamed fibrous stroma in which the characteristic diagnostic features were destroyed, and cases reported with a connective tissue disease were excluded.

A total of forty-two cases were selected for this study, twenty cases of formalin-fixed paraffin-embedded (FFPE) blocks each of twenty OKC (n=20), and twenty AB (n=20), and two cases of DC as controls.

Tissue processing and staining

From all selected forty-two FFPE blocks two sections of 4 µ thickness were taken, of which one was stained with hematoxylin and eosin (H & E) following the standard protocol, and then observed under a conventional light microscope to reassess diagnosis, and to be subjected to the selection criteria. The other section was stained with picrosirius red, the staining kit was purchased from Bio-Optica Milano S.p.A under the code number 04-121873 and LOT 0420, the kit contained four bottles, A: sirius red picrate solution, B: buffer solution (two in number), and C: Mayer's Hemalum.

Staining procedure: sections were brought to distilled water, and then put on 10 drops of **A** and left to act 50 minutes. Sections were rinsed briefly in distilled water, and put on 10 drops of reagent **B** which was left to act 2 minutes and repeated 2 times. Sections were briefly rinsed in distilled water and slides were drained. 10 drops of reagent C were used and left to act 3 minutes. Sections were blued in running tap water for 3 minutes, then dehydrated through ascending alcohols, and cleared in xylene. Sections were then viewed under a polarized light microscope for the analysis of collagen fibers.

Evaluation of collagen fibers in OKC and AB cases

The analysis of all selected cases stained with PSR was performed under a polarizing microscope using 10x and 40x magnifications in at least three separate fields.

Polarization colors of collagen fibers

The connective tissue of the two lesions showed polarization colors varying from green-yellow, yellow-orange and orange-red. The most predominant polarization color was determined in all cases, and this was performed during three separate periods of times to eliminate intra-observer bias, and the average reading for each case was recorded.

Orientation of collagen fibers

Collagen fibers orientation to the epithelial component was also observed, each case was classified according to the fibers orientation pattern as not parallel or parallel.

Organization of collagen fibers

The organization of collagen fibers was also evaluated in areas near to the epithelial component, and each case was classified as loose bundles of collagen fibers (loosely arranged and interwoven in all directions), or dense collagen fibers (well-defined organization with orderly organized collagen fibers forming collagen lamellae). All information obtained were documented in a chart for each case.

Statistical analysis

All obtained information was analyzed using SPSS format version 20 (Statistical Package for Social Sciences, Chicago, Illinois, USA) for statistical analysis. The polarization colors, orientation and organization of collagen fiber bundles in the two lesions were tested using Fisher's exact test and Chi-square test. Computations of P-value (<0.05) considered as statistically significant.

RESULTS

Polarization colors of collagen fiber bundles in the connective tissue of OKC and ameloblastoma

On evaluating PSR stained sections of control cases of DC they showed a predominance of orange-red and yellow-orange birefringence. OKC and AB cases revealed the following distribution of polarization colors of collagen fibers: green-yellow birefringence was the most predominant in OKC cases, and it occupied twelve (60%), followed by yellow-orange which was recorded in seven cases

(35%), orange-red was documented in only one case (5%) (Figure 1,2).

On the contrary, most of the collagen fibers in AB cases exhibited a predominance of yellow-orange birefringence in eleven (55%), the green-yellow was documented in five cases (25%), and the orange-red color was noticed in four cases (20%) (Figure 3,4), (Table 1). (p-value= 0.086).

Figure 1 photomicrograph of PSR stained section of OKC showing a predominance of green-yellow birefringence of collagen fibers. 10x

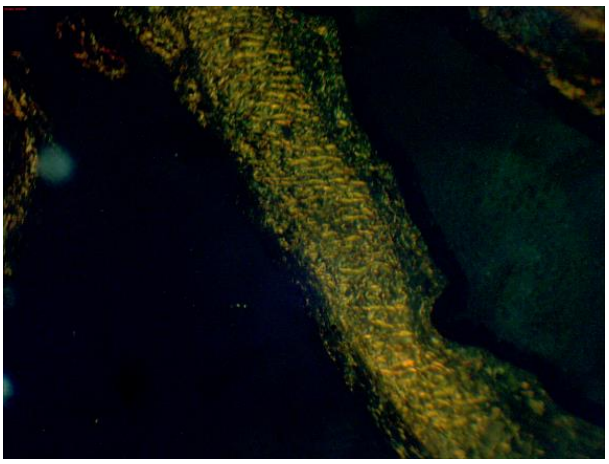


Figure2 photomicrograph of PSR stained section of OKC showing a predominance of green-yellow birefringence of collagen fibers.40x

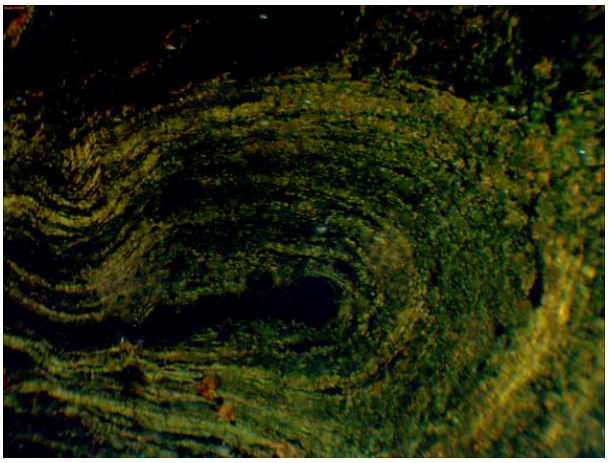


Figure 3 photomicrograph of PSR stained section of ameloblastoma showing a predominance of yellow-orange birefringence of collagen fibers. 10x

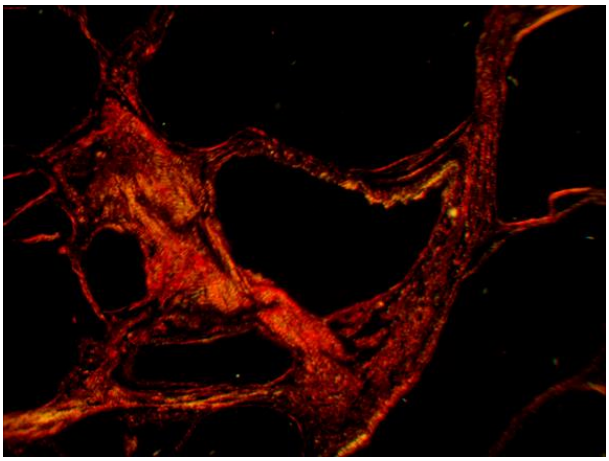


Figure 4 photomicrograph of PSR stained section of ameloblastoma showing a predominance of yellow-orange birefringence of collagen fibers. 40x

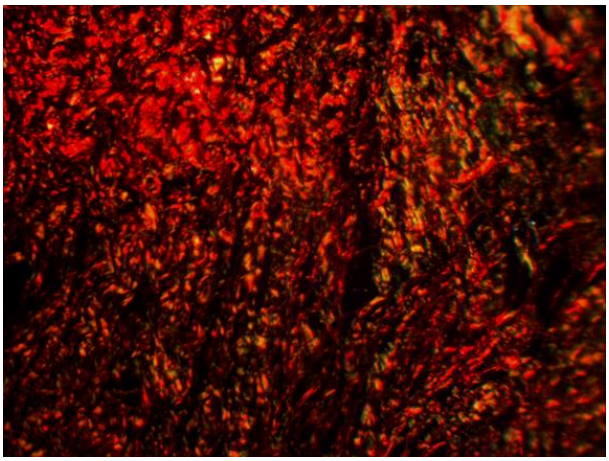


Table 1: comparison of polarization colors of collagen fiber bundles in the stroma of OKC and ameloblastoma

Collagen	OKC		AB	
	N	%	N	%
GY	12	60	5	25
YO	7	35	11	55
OR	1	5	4	20
Total	20	100%	20	100%

(*p-value= .086) (OKC: odontogenic keratocyst, AB: ameloblastoma, GY: green-yellow, YO: yellow-orange, OR: orange-red).

***The orientation of collagen fiber bundles to the epithelial component in OKC and ameloblastoma*:**

The distribution of collagen fibers orientation pattern was the same in both OKC and AB cases, fibers were predominantly not parallel in eleven cases (55%), while parallel fibers were seen in nine cases (45%), (Figure 5,Table 2) (p-value=1.00).

Figure 5 photomicrograph of collagen fibers running parallel to the epithelial component in an OKC, 10x

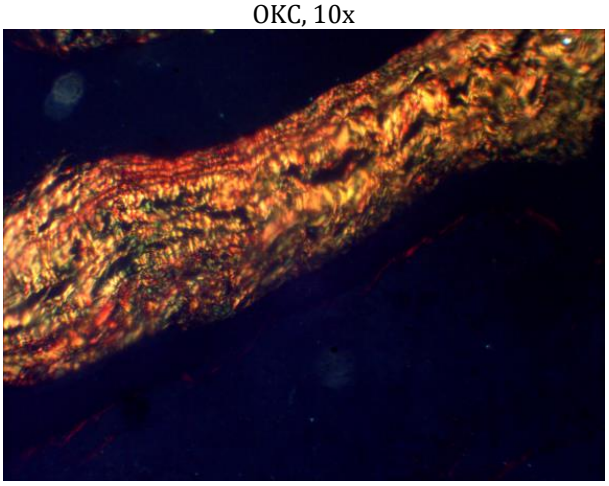


Table 2: comparison of orientation pattern of collagen fiber bundles in the of OKC and ameloblastoma

Collagen	OKC		AB	
	N	%	N	%
Not parallel	11	55	11	55
Parallel	9	45	9	45
Total	20	100%	20	100%

(*p-value=1.00)(OKC: odontogenic keratocyst, AB: ameloblastoma)

***Organization pattern of collagen fiber bundles in the connective tissue of OKC and ameloblastoma*:**

Concerning the organization of collagen fibers it was demonstrated that, densely packed collagen fiber bundles were predominant in both OKC and AB accounting for eleven (55%) and twelve (60%) respectively; however, loosely packed fibers were documented in nine cases (45%) of OKC and eight cases (40%) of AB, (Figure 6, Table 3). (p-value=0.074)

Figure 6 photomicrograph of PSR stained section of ameloblastoma showing densely packed collagen fibers, 10x

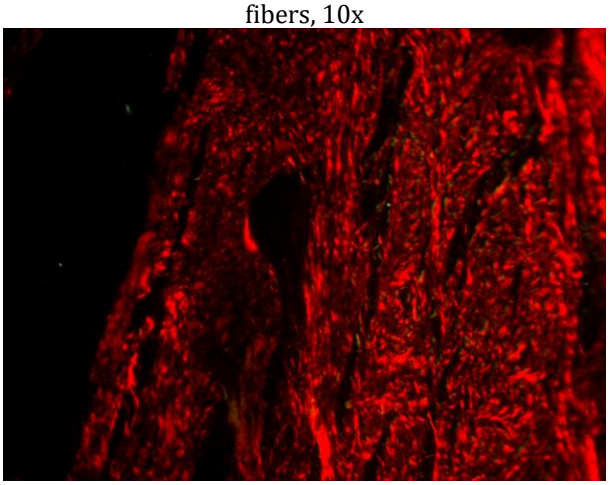


Table 3: comparison of organization pattern of collagen fiber bundles in the stroma of OKC and ameloblastoma

Collagen	OKC		AB	
	N	%	N	%
Dense	11	55	12	60
Loose	9	45	8	40
Total	20	100%	20	100%

(*p-value=.749) (OKC: odontogenic keratocyst, AB: Ameloblastoma).

DISCUSSION

This study was conducted in an attempt to assess, compare and investigate the difference in the polarization colors of collagen fiber bundles, and their pattern of orientation and organization in the connective tissue of OKCs and ameloblastoma with reference to their biologic behavior. This was a histochemical study, containing a total of forty two FFPE blocks of pre-diagnosed cases of OKC and ameloblastoma , twenty cases each, and two cases of DC were taken as controls, the sample selection was made upon inclusion and exclusion criteria.

Sections of 4µ were stained with PSR, and then viewed under a polarized light microscope using 10x and 40x magnifications in at least three separate fields; first, to record the most predominant polarization color the examination was conducted during three separate periods of times to eliminate intra-observer bias, and the average reading was taken for each case; second, patterns of orientation and organization of collagen bundles in each case were documented.

It was proposed that PSR staining and polarized light microscopy can effectively help to define the nature and arrangement of collagen fibers in odontogenic lesions, as mature thick well-packed

fibers demonstrate orange-red to red birefringence while immature thin loose fiber show a birefringence of green-yellow color.[5] It has been documented that, the study of collagen fibers nature and the overall impact of connective tissue on the behavior of the OKC and ameloblastoma can help to explain the mechanism of expansion and reasons behind their aggressive growth and high recurrence rate.[6]

In the present study, it was observed that twelve cases (60%) of OKCs exhibited a predominance of green-yellow birefringence, this was in accordance with other several studies.[5,10-13] Green-yellow polarization is related to loosely packed collagen fibers, this is in agreement with Sharf *et al.*[14] who advocated that, physical aggregation of collagen molecules determine the polarization color; therefore, it was found that loosely packed collagens represented as green-yellow, this is also in conjunction with Junqueira *et al*., and Montes *et al*.[12] who documented that, loose thin fibrils of type III collagen are found to physically form a loose meshwork in the ground substance which in turn exhibit a weak birefringence of green-yellow.[15]

It was also stated that, green-yellow color implies that stromal fibers consist of pro-collagen, intermediate, or pathologic collagens with disorganized pattern, such fibers are not structurally stable; hence, denature easily by the action of proteolytic enzymes of the stromal tissue. It has been shown that abnormal or immature fibers are found to be associated with aggressive lesions.[5,12,16]

On the other hand, ABs demonstrated green-yellow birefringence in five cases (25%) which is less than OKC, this does not apply with reference to their biologic behavior as AB is an odontogenic tumor whereas OKC has been re-classified as an odontogenic cyst.[17] Our finding contradicts the findings of Peddapeli K *et al*.[16] in which AB showed slightly more of green-yellow color than the OKC.

Moreover, AB displayed more yellow-orange and orange-red birefringence than OKC cases, eleven (55%) and four (20%) compared to seven (35%) and one (5%) respectively. Our finding is in agreement with other studies.[10,14,18] It was proposed that strong birefringence of orange and red denotes tightly packed collagens, and this is compatible with Junqueira *et al*.[14] who claimed that, type I collagen are thick and closely packed; thus, fibers react strongly with PSR stain and produce long wavelength of orange and red color when viewed under PL.[12,15]

On the contrary, Peddapeli K *et al*.[16] reported that, OKCs showed more yellow-orange and orange-red when compared to ABs**.** Our findings can be justified as we have not included moderately and severely inflamed cases of OKC in an attempt to study this developmental lesion without the recognized alteration on collagen fibers by secondary inflammation as evaluated by Kajikar MS *et al*.[5], it was shown that infected OKCs revealing a severely inflamed stroma demonstrated orange to red birefringence; unlike non-infected OKCs which illustrated a predominance of green-yellow.

Also, it was reported that inflammation can induce a change in collagen fibers organization in inflamed OKCs by increasing the production of densely packed fibers; therefore, influence a change in the color of polarization.[19] It has been documented that strongly birefrengent collagen fibers have been correlated to odontogenic lesions with innocent behavior and less aggressive growth.[12,13]

In the present study, the observations made on collagen fibers orientation showed equal cases of OKCs and ABs in which parallel fibers were reported in nine (45%) and not parallel in eleven (55%). Our findings were conflicting with the findings of other studies in which parallel fibers were predominant in OKC cases.[16-20] Parallel orientation has been considered to contribute to the separation between lining epithelium of OKCs from their supporting capsule, this was also linked to the different growth pattern of OKC from other odontogenic cysts, which in addition to daughter cysts explained the unceasing growth and high recurrence of the OKC.[12,16]

Our study revealed that, loose collagen fibers distribution in OKCs was insignificantly more than ABs, nine (45%) compared to eight cases (40%) respectively. This is in agreement with Peddapeli K *et al.[16]*, it has been demonstrated that loosely packed collagen fibers are prone to degradation by collagenases; as a result, facilitating the growth and expansion of a lesion. Moreover, studies on the role of connective tissue of OKC advocated that, the function of the fibrous capsule of this lesion extends beyond structural support; it is rather involved in its pathogenesis and clinical behavior.[5,16]

Conversely, dense collagen fibers in ABs were seen in twelve (60%) compared to eleven (55%) in OKCs, this can be attributed to the presence of mature densely packed fibers of long-standing lesions, this is consistent with the findings of Aggarwal P *et al*.[21] However, our findings showed a contradiction with other studies.[18-20] It was shown that, cytokines and growth factors regulate the process of collagen and ECM components synthesis and degradation; in addition to defining the architecture of collagens within a tissue by influencing the activity of fibroblasts causing the production of thick mature fibers.[15] Hence, collagen matures as the content of its proteoglycans and water decreases; subsequently, there is an increase in the fiber diameter with evidence of densely packed fibers organization.16

According to the observations made on the differences in the polarization colors, orientation and organization of collagen bundles in the connective tissue of OKC and AB they were found to be statistically insignificant (p-value=.086,1.00,.749 respectively). This was consistent with Raj Y. *et al*.[5] and Ali AN *et al*.[20] However, our findings were conflicting with Peddapeli K *et al*.[16] in which a significant difference was documented only between the means of yellow-orange; in addition, a difference was found in the orientation and organization of collagen fibers in OKC and AB.

This study showed some limitations, these limitations are:

- Selection bias, the study sample was not randomly chosen.
- Small sample size; hence, no firm conclusions can be drawn from this sample as a significant difference would be difficult to identify.
- Limited prior research on the same topic.
- Time constrains due to the current pandemic situation.
- Funding issues.
- Limited access to the literature.

Despite constrains, this research has shown a potential significance:

- Cases that were only applying to the diagnostic criteria of OKC and ameloblastoma were included; moreover, altered connective tissues by inflammation were excluded as inflammation dose not play a role in the pathogenesis of theses lesions.
- This research is filling the gap in the literature as being one of the few comparing the OKC and ameloblastoma in terms of collagen fibers characteristics.
- Despite the modest size of the sample, this sample can be considered relatively larger than the samples of other studies investigating the differences of collagen fibers among odontogenic lesions.
- Examination of all cases was performed in at least three different fields for three times separately to eliminate intra-observer bias and to insure accuracy of determining the most predominant color in each case.

CONCLUSION

- PSR staining and polarizing microscopy is a powerful tool to appraise the nature and arrangement of collagen fibers in odontogenic lesions.
- The connective tissue of OKC showed a predominance green-yellow birefringence, and loosely packed fibers, this can be correlated to the aggressive nature of this lesion.
- Studying and assessing collagen fibers of odontogenic lesions, especially lesions of diagnostic significance, may help to predict the clinical course of these lesions.

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