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RESEARCH ARTICLE

ASSESSMENT OF COLLAGEN IN ODONTOGENIC LESIONS: A HISTOCHEMICAL STUDY USING PICROSIRIUS RED STAIN AND POLARIZING MICROSCOPY

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ABSTRACT

Background: Variations in structure, maturation, and arrangement patterns in odontogenic lesions, affect clinical and biological behavior.
Aim: The aim was to assess arrangement and distribution of collagen fibers in Odontogenic lesions histochemically using Picrosirius Red Stain and observed under Polarizing Microscopy.
Materials and Methods: Samples included formalin fixed-paraffin embedded tissue from 10 dental follicular tissue, 30 odontogenic cysts: 15 Radicular cysts; 15 Dentigerous cysts; 30 benign odontogenic tumors: 15 keratocystic odontogenic tumors, 15 ameloblastoma and 5 ameloblastic carcinoma. Sections stained with picrosirius red stain were examined under polarizing microscope (40x) for thickness, birefringence and orientation patterns.
Statistical analysis: Data were analyzed using chi-square test, one-way ANOVA and Student's t-test for 'pair-wise' comparisons. P-value ≥ 0.05 was considered significant.
Results: Dental follicular tissue and odontogenic cysts showed thick fibers with orange red birefringence and reticular pattern. Radicular cysts showed higher percentage of thick collagen fibers, orange red birefringence and reticular pattern. Keratocystic odontogenic tumor and ameloblastoma showed thick collagen fibers and parallel pattern. Ameloblastic carcinoma showed thick fibers with orange red birefringence and reticular pattern.
Conclusion: Collagen fibers as studied by picrosirius red stain and polarizing microscopy may be a useful tool in assessing the nature, aggressiveness and growth potential of these lesions.
Clinical significance: The nature of collagen fibers in the connective tissue stroma may be evaluated by picrosirius red stain and polarizing microsopy, may be a useful tool in assessing the nature, aggressiveness and growth potential of these lesions.

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INTRODUCTION

Tissues contributing to odontogenesis undergo stages of differentiation with the time. Thus, any of the tissues participating in this process may be involved in the development of various pathologies either in cysts, hamartomas or tumors which are collectively often referred to as 'odontogenic cysts' and odontogenic tumors' (Prabhu *et al*., 1992). Odontogenic cysts are probably the most common destructive lesions of the jaw bones.

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The dentigerous cyst originates through alteration of reduced enamel epithelium after the crown of a tooth has been completely formed and is considered as developmental cysts whereas radicular cysts which develop from cell rests of Malassez as a result of bacterial infection and pulpal necrosis in carious teeth, are inflammatory in nature. Pathologic alterations in remnants of odontogenic epithelium and odontogenic mesenchyme may lead to the formation of a variety of tumors called odontogenic tumors. These may form because of alterations in the odontogenic epithelium, mesenchyme or both. Depending upon this, there are variations in the connective tissue component of these odontogenic tumors. Benign epithelial odontogenic tumors in this study, include ameloblastoma and keratocystic odontogenic tumor

whereas malignant epithelial odontogenic tumors comprised of 'ameloblastic carcinoma'. Collagen fibers, forming an important component of the connective tissue, may vary in their structure, maturation and may eventually affect the biological behavior of the neoplasm. Collagen constitutes 34% of the extracellular matrix proteins and form an integral part of connective tissue stroma. It plays a vital role in maintaining the structural integrity and in determining tissue function (Aggarwal *et al*., 2011; Rich, 2005). Collagen has a natural birefringence property which is attributed to the arrangement of its fibers which can be studied by special stains such as Van Gieson, Masson's trichrome and Picrosirius red stain (Constantine, 1968). Though, Van Gieson and Masson's trichrome stain may not be the ideal stains for the detection of collagen fibers as both these methods fail to reveal thin collagen fibers. This drawback of these stains incited Puchtler and colleagues to seek a better compound. They discovered that Sirius red F3BA (color index 135780) dissolved in a saturated picric acid solution (picrosirius red) consistently stained thin collagen fibers, did not fade for a long time and was appropriate for use with polarized microscopy (Rich, 2005). Examination of collagen fibers by Picrosirius red stain in conjunction with polarizing microscope can serve as a useful procedure to differentiate procollagen, intermediate and pathological collagen fibers, from normal fibers (Vij *et al*., 2011). The aim of this study is to assess the arrangement and distribution of collagen fibers in Odontogenic lesions (Odontogenic cysts and tumors) histochemically by using Picrosirius Red Stain and Polarizing Microscopy.

MATERIALS AND METHODS

The study sample included 75 formalin-fixed paraffin embedded tissue blocks previously diagnosed, which were obtained from the archives of the Department of Oral and Maxillofacial Pathology. The samples were grouped as follows: Control Group comprised of 10 normal dental follicular tissues, 30 cases of Odontogenic cysts (Group I) which included 15 cases of Radicular cysts (Group IA) and 15 cases of Dentigerous cysts (Group IB), 30 cases of Benign odontogenic tumors (Group II), included 15 cases of Keratocystic odontogenic tumors (Group IIA) and 15 cases of Ameloblastoma (Group IIB). Malignant odontogenic tumors (Group III) comprised of 5 cases of Ameloblastic carcinoma (Graph1). The tissue samples were sectioned at 5µm thickness and transferred on to egg albumin coated slides. After deparaffinization and hydration in distilled water, the sections were stained with Weigert's haematoxylin for 8 minutes and washed under running tap water. This was followed by incubating the sections in 0.1% (w/v) Sirius red F3B(Direct red 80, 365548-5G, dye content 25% Sigma Aldrich, St. Louis, US, dye content 25%) in saturated aqueous solution of picric acid (32035, s.d. fine-CHEM Limited, Mumbai, India) for 1 hour at room temperature. The sections were differentiated in acidified water and dehydrated in absolute alcohol and DPX mounted. The stained sections were examined and evaluated under polarizing Nikon Research Microscope (ECLIPSE 80i) and photographed using CCD Video Camera (NIKON DS-U2, 5.03). Image analysis software: NIS-ELEMENT, BASIC RESEARCH, VERSION 2.32 (Windows XP) in which the calibrations were set in micrometer for assessing diameter of collagen fibers. Criteria for assessment of collagen fibers: Three parameters of collagen fibers were evaluated based on thickness, birefringence color and arrangement patterns. For thickness evaluation, collagen fibers were grouped into thin and thick fibers. Fibers with thickness measuring 0.8µm or less were classified under 'thin' fibers and fibers thickness measuring between1.4µm to 2.4µm was classified under 'thick' fibers. Fiber thickness was measured under 40x magnifications by analyzing, ten alternate high power fields in connective tissue. Based on birefringence, collagen fibers were grouped into orange-red and yellow-green birefringence and for the orientation patterns, into reticular and parallel pattern. Same fields were assessed for thickness, birefringence color (yellow-green and orange-red) and orientation pattern (reticular and parallel) of collagen fibers. Their quantitative assessment was done in the mean percentage values of predominance of thick and thin fibers, yellow-green and orange-red birefringence and reticular and parallel orientation pattern of collagen fibers. Collagen fibers orientation was observed in relation to the epithelial component. The data obtained was stored and tabulated for statistical analysis using SPSS VERSION 13.0 for all the groups and subgroups. The data obtained were subjected to ANOVA for multiple comparisons, paired t-test for pairwise comparisons and chi-square test for categorical analysis. 'P' value < 0.05 was considered as 'statistically significant' while P value < 0.001 was considered as 'highly significant'.

RESULTS

Based on thickness

Radicular cysts showed statistically predominant thick fibers in 93% of the cases and 60% cases of the Dentigerous cysts (Table 1) (Fig 1, 2). When the two odontogenic cysts (Radicular cyst and Dentigerous cyst) were compared, difference in the thickness was found statistically nonsignificant (P=0.43) (Table 2). Keratocystic odontogenic tumor in 87% cases showed statistically predominant thick collagen fibers where as Ameloblastoma showed predominantly thick fibers in 67% of the cases (Table 1) (Fig 3, 4). This difference in thickness between these two tumors was found to be statistically nonsignificant (P=0.088) (Table 2). Malignant odontogenic tumors (Ameloblastic carcinoma) showed predominantly thick fibers in all the cases (P=0.003) (Table 3) (Fig 5).

Based on birefringence color

Radicular cysts showed orange-red birefringence in 87% cases whereas dentigerous cysts showed predominantly, orange red birefringence in 67% of cases (Table 1) (fig 1, 2). When the two odontogenic cysts (Radicular and Dentigerous cysts) were compared, difference in the birefringence color of collagen fibers between these two subgroups were found statistically insignificant (P=0.82) (Table 2). Keratocystic odontogenic tumor in 87% cases showed predominant orange red birefringence, whereas, Ameloblastoma in 73% of the cases showed predominantly yellow green birefringence (Table 1) (Fig 3,4). On comparing the two benign odontogenic tumors, the difference in the birefringence colors was found to be significant (P=0.016) (Table 2). Malignant odontogenic tumors (Ameloblastic carcinoma) showed predominantly orange red birefringence in 100% of the cases (Table 1) (P=0.007) (Table 3) (Fig 5).

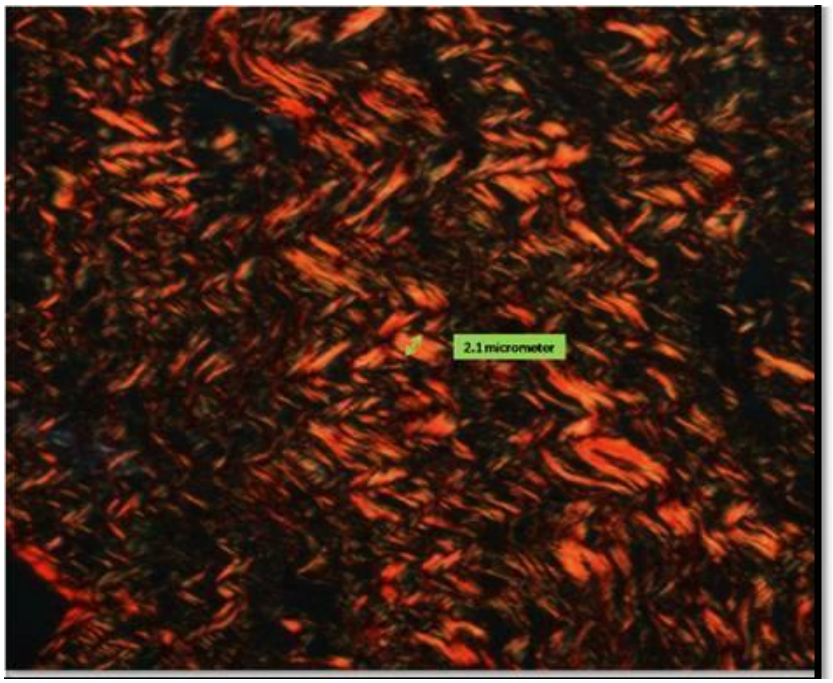

Fig. 1. Predominantly thick fibers and orange red birefringence and reticular orientation pattern of collagen fibers in the Group IA (Radicular cyst)

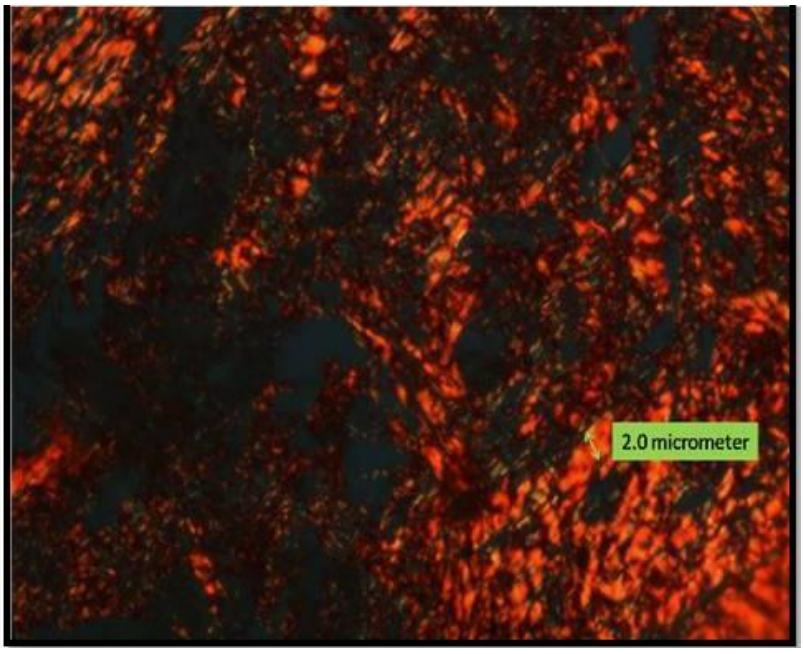

Fig. 2. Predominantly thick fibers and orange red birefringence and reticular orientation pattern of collagen fibers in the Group IB (Dentigerous cyst)

Table 1. Distribution of cases according to thickness, bireringence and orientation of collagen fibers in groups

Parameter	Predominance	Radicular cysts [no of cases (%age)]	Dentigerous cysts [no of cases (%age)]	KCOT [no of cases (%age)]	Ameloblastoma [no of cases(%age)]	Ameloblastic carcinoma [no of cases (%age)]
Thickness	Predominantly thick fibers	14(93)	9(60)	13(87)	10(67)	5(100)
	Predominantly thin fibers	1(7)	6(40)	2(13)	5(33)	0(00)
Birefringence	Predominantly yellow green	2(13)	5(33)	2(13)	11(73)	0(000
	Predominantly orange red	13(87)	10(67)	13(87)	4(27)	5(100)
Orientation	Predominantly reticular	14(93)	10(67)	2(13)	2(13)	3(60)
	Predominantly parallel	1(7)	5(33)	13(87)	13(87)	2(40)

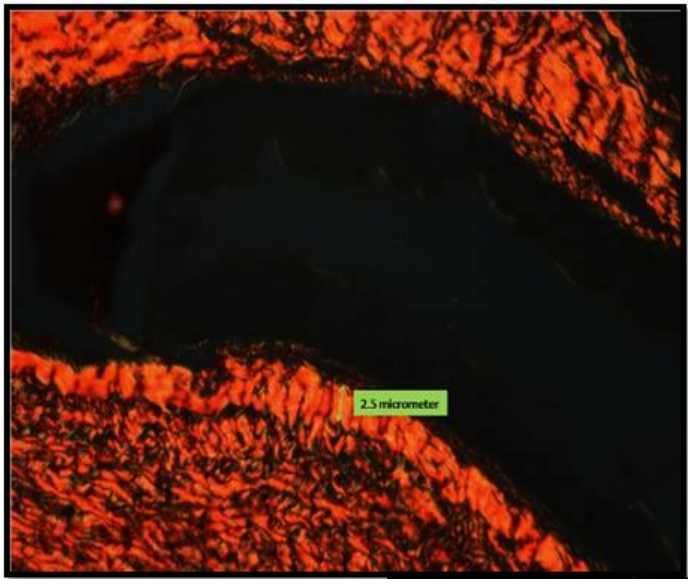

Fig. 3. Predominantly thick fibers and orange red birefringence and parallel orientation pattern of collagen fibers in the Group IIA (Keratocystic odontogenic tumor)

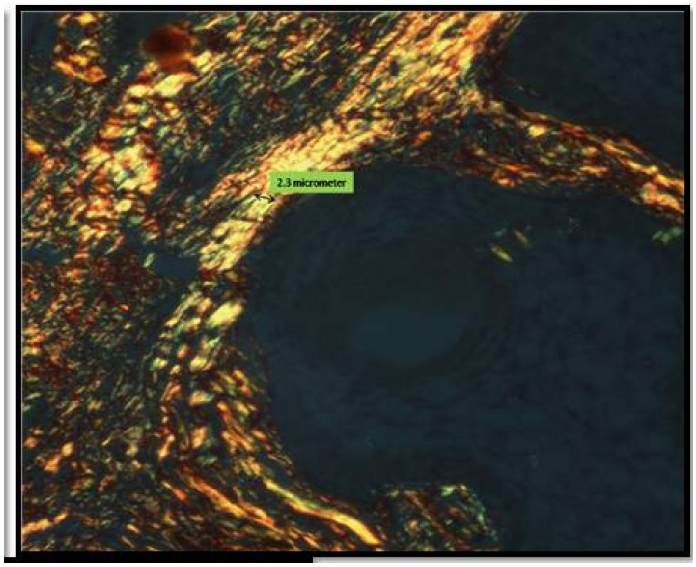

Fig. 4. Predominantly thick fibers and yellow green birefringence and parallel orientation pattern of collagen fibers in the Group IIB (Ameloblastoma)

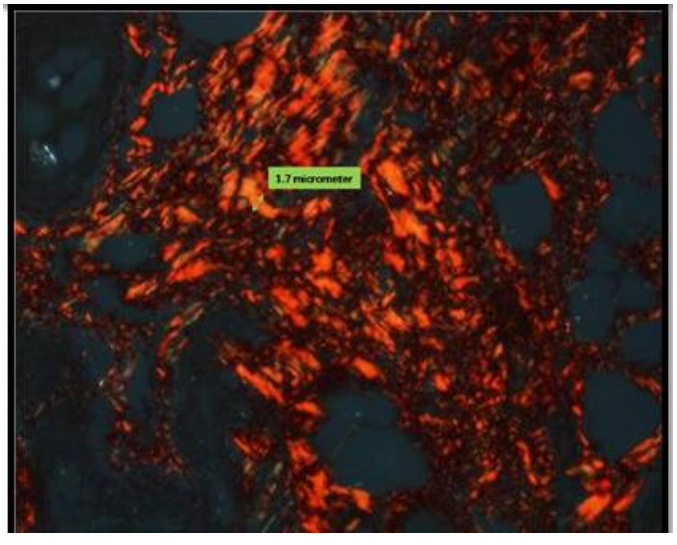

Fig. 5. Predominantly thick fibers and orange red birefringence and reticular orientation pattern of collagen fibers in the Group III (Ameloblastic carcinoma)

Based on the orientation pattern of collagen fibers

93.0% cases of radicular cysts and 67% cases of dentigerous cysts showed predominantly reticular orientation pattern (Table 1) (Fig 1,2). On comparing the odontogenic cysts (Radicular cyst and Dentigerous cyst) predominance of reticular pattern was found statistically highly significant (P=0.009) (Table 2). Keratocystic odontogenic tumors and Ameloblastoma, in 87% of the cases showed parallel orientation of fibers with only 13% showing predominant reticular pattern (Table 1) (Fig 3,4). This predominance of parallel arrangement was found to be statistically insignificant (P=0.60) (Table 2). Malignant odontogenic tumors (Ameloblastic carcinoma) showed reticular pattern in the 60% of the cases (Table 1) (Fig 5). On comparing Ameloblastoma with Ameloblastic carcinoma, statistically significant (P=0.01) difference in the orientation pattern of collagen fibers was found (Table 2).

Table 2. Comparison between various subgroups

Parameters		Radicular cyst-dentigerous cyt	KCOT-ameloblastoma	Ameloblastoma-ameloblastic carcinoma
Thickness	t-value	0.789	1.766	-3.741
	P value	0.437	0.088	0.009**
Birefringence	t-value	0.225	2.553	4.766
	P value	0.823	0.016*	0.002**
Orientation	t-value	2.792	0.517	-2.238
	P value	0.009**	0.609	0.016*

Table 3. Assessment of collagen fibers among groups based on their mean percentage value

Paramet er		Mean±standard deviation					
		Conrol gr	Gr I A(radicular cyst)	Gr I B(Dentigerous cyst)	Gr II A(KCOT)	Gr II B (Ameloblastoma)	Gr III(Ameloblastic carcinoma)
Thickness	Thick	58.68 ± 15.94	66.76±18.09	61.42±19.01	66.79±14.64	56.56±16.98	80.30±10.24
	Thin	41.32 ± 15.94	33.20±18.10	38.58±19.01	33.20±14.64	43.43±16.98	19.70±10.24
	Thick-thin(p-value)	0.119 (t = 1.721)	0.003** (t=3.590)	0.036* (t=-2.326)	0.001** (t=4.440)	0.157 (t=1.497)	0.003** (t=6.611)
Birefringence	Yellow green	43.24±15.26	35.43±15.16	36.74±16.59	40.64±19.04	57.91±17.99	27.50±9.77
	Orange red	56.75±15.26	64.56±15.16	63.25±16.59	59.35±19.04	42.08±17.99	72.49±9.77
	YG-OR(p-value)	0.195 (t=-1.399)	0.002** (t=3.718)	0.008** (t=3.093)	0.078(t=-1.903)	0.110(t=1.704)	0.007** (t=-5.145)
Orientation	Reticular	72.05±21.47	81.42±25.10	51.76±32.59	26.13±21.64	21.18±30.07	66.38±41.67
	Parallel	27.95±21.47	18.57±25.10	48.23±32.59	73.86±21.64	78.80±30.07	33.62±41.67
	Reticular-parallel(p-value)	0.010* (t=3.247)	0.001** (t=4.848)	0.837 (t=0.210)	0.001** (t=-4.270)	0.002** (t=3.710)	0.429 (t=-0.879)

DISCUSSION

Dental follicle is a structure that surrounds unerupted teeth and is composed of fibrous connective tissue which contains remnants of odontogenic epithelium with proliferative potential may account for the predominance of thick orange red collagen fibers in the present study (Moure *et al*., 2011). Control tissue (normal follicular tissue) showed thick fibers with orange red birefringence and reticular pattern. Similar results were also given by Singh *et al* (2012) and Moure *et al* (2011) who demonstrated dense thick collagen fibers in the dental follicle, supporting the present study (Moure *et al*., 2011; Singh *et al*., 2012). Hirshberg *et al*. (1996) showed more orange red birefringence of thick fibers of hyperplastic dental follicle. Collagen fibers were oriented in reticular pattern predominantly which may account for its growth. Moure *et al*. (2011) also demonstrated collagen fibers with interwoven arrangement in their study.Inflammation releases various cytokines and growth factors which cause proliferation of fibroblasts and extracellular matrix which result in formation of thickened mature collagen.

During collagen fibers maturation, change in proteoglycan content of fibers causes dehydration of fibers resulting in increase in diameter of collagen fibers (Vij *et al*., 2011). This suggests that inflammation in Radicular cysts may be responsible for the presence of tightly packed thick orange red collagen fibers. This finding goes in accordance with a study of done by Vij *et al.* (2011) in which closely packed thick mature collagen fibers were observed in Radicular cysts. The predominant reticular pattern of collagen fibers may attribute to the expansion and growth of these cysts. Dentigerous cyst, as being developmental in origin, their long standing nature might be the cause of closely packed collagen fibers accounting for its predominant orange red birefringence. These results were in accordance with Hirshberg *et al.* (1999) who in their study showed that tightly packed thick yellowish-orange collagen fibers were predominant in Dentigerous cysts.

Also, Moure *et al*. (2011) found presence of thick fibers arranged in dense bundles, throughout the connective tissue wall of Dentigerous cyst without any inflammation and thin collagen fibers, in Dentigerous cysts with the inflammatory infiltrate and no parallel pattern of fiber orientation. A predominant reticular pattern of collagen fibers suggests that dentigerous cyst is a reactive tissue, which induces an expansile growth associated with fluid accumulation. Similar results were observed by Singh *et al.* (2012) in their study who demonstrated predominant nonparallel pattern of collagen fibers in Dentigerous cysts. Thus, dentigerous cyst showed a similar pattern of collagen fibers, which, when assessed were similar to dental follicle. This may be associated with the pathogenesis of this lesion as supported by the intrafollicular theory of origin of dentigerous cyst, which suggests that these entities may develop by accumulation of fluid either between reduced enamel epithelium or within the enamel organ itself. Among the Benign odontogenic tumors, the connective tissue stroma of Keratocystic odontogenic tumor represents mature thick collagen fibers accounting for its orange-red birefringence under polarizing microscopy. The results of the

present study are in accordance with Aggarwal and Saxena (2011) who also observed predominant orange red birefringence indicating closely packed collagen fibers in Keratocystic odontogenic tumors. The predominant parallel arrangement of collagen fibers may be associated with neoplastic behavior of Keratocystic odontogenic tumor; which may also act as an additional factor in facilitating the separation of the epithelial lining from the underlying connective tissue wall, accounting for its recurrence potential. Singh *et al.* (2012) also demonstrated parallel arrangement of collagen fibers in Keratocystic odontogenic tumor in their study on the qualitative and quantitative comparative analysis of collagen fibers to determine the role of connective tissue stroma in its biological behaviour. The influence of inflammation on the packing of collagen fibers in the connective tissue wall of Keratocystic odontogenic tumor was investigated by Hirshberg *et al.* (2007). Hence, from the result of the present study it may be inferred that the function of the stroma of Keratocystic odontogenic tumor could possibly be regarded as a structural support but also as playing a part in their neoplastic behaviour.

In the current study, Ameloblastoma showed predominantly thick fibers with yellow green birefringence and parallel arrangement of collagen fibers thus, ameloblastoma is composed of thick procollagen fibers with parallel arrangement around the epithelial component and in the subepithelial ectomesenchyme which accounts for its neoplastic growth pattern. The Unicystic ameloblastoma also showed predominant yellow green birefringence in the sub epithelial areas of the fibrous capsule which give support to the idea that it should be regarded as a cystic neoplasm. This result goes in accordance with the study done by Zhang *et al.* (2011). The malignant odontogenic tumor (ameloblastic carcinoma) showed extensively thick collagen fibers with orange red birefringence and predominantly reticular pattern. This may suggest that the connective tissue stroma of ameloblastic carcinoma is composed of thick mature collagen fibers. The growth of the lesion may be attributed to its reticular orientation pattern of collagen fibers. Till date no studies have been done on ameloblastic carcinoma for the assessment of collagen fibers in the connective tissue stroma for comparing the biological behavior of these entities.

Conclusion

Connective tissue stroma of these odontogenic lesions may account for their varied biological behavior. The mature collagen fibers appeared thick in diameter and had orange red birefringence predominantly; on the other hand the immature fibers were thin and exhibited yellow green birefringence. The cysts showed thick, orange red and reticular patterns in collagen arrangement, whereas, the odontogenic tumors showed showed parallel arrangement and thick fibers. Odontogenic keratocyst showed orange red where as ameloblastoma exhibited yellow green birefringence predominantly. Ameloblastic carcinoma exhibited thick fibers in reticular arrangement and orange red birefringence. Thus it can be opined that odontogenic cysts have a potential of behaving as aggressive as ameloblastic carcinoma. Hence, should be managed with caution.

Clinical significance

The nature of collagen fibers in the connective tissue stroma may be evaluated by picrosirius red stain and polarizing microsopy, may be a useful tool in assessing the nature, aggressiveness and growth potential of these lesions. Also, if applied to other pathological conditions, this technique may help in predicting their nature in terms of biological behavior and prognosis.

REFERENCES

Aggarwal, P. and Saxena, S. 2011. Stromal differences in odontogenic cysts of a common histopathogenesis but with different biological behaviour: A study with picrosirius red and polarizing microscopy. *Indian J Cancer,* 48(2): 211-215.

Constantine, V.S. and Mowry, R.W. 1968. Selective staining of Human Dermal Collagen II. The use of picrosirius red F3BA with polarization microscopy. *J Invest Dermatol,* 50:419-423.

Hirshberg, A., Buchner, A. and Dayan, D. 1996. The Central Odontogenic Fibroma and hyperplastic dental follicle: study with picrosirius red and polarizing microscopy. *J Oral Pathol Med.,*25: 125-127.

Hirshberg, A., Lib, M., Kozlovsky, A. and Kaplan, I. 2007. The influence of inflammation on the polarization colors of collagen fibers in the wall of Odontogenic keratocyst. *Oral Oncol.,* 43: 278-282.

Hirshberg, A., Sherman, S., Buchner, A. and Dayan, D. 1999. Collagen fibers in the wall of odontogenic keratocysts: a study with picrosirius red and polarizing microscopy. *J Oral Pathol Med.,* 28:410-412.

Moure, S.P., Carrard, V.C., Lauxmen, I.S.A., Manso, P.P., Oliviera, M.G., Martins, M.D. *et al*. 2011. Collagen and elastic fibers in odontogenic entities: analysis using light and confocal laser microscopic methods. *The Open Dentistry Journal,* 5:116-121.

Prabhu, S.R., Wilson, D.F., Daftary, D.K. and Johnson, N.W. 1992. Oral Diseases in the Tropics, 1st ed, Oxford University Press, India, p.367.

Rich, L. and Whittaker, P. 2005. Collagen and picrosirius red staining: A polarized light assessment of fibrillar hue and spatial distribution. *Braz J Morphol Sci.,* 22(2): 97-104.

Singh, H.P., Shetty, D.C., Wadhwan, V. and Aggarwal, P. 2012. A Quantitative and Qualitative comparative analysis of collagen fibers to determine the role of collagen fibers to determine the role connective tissue stroma on biological behavior of Odontogenic cyst: A histochemical study. *Natl J Maxillofac Surg.,* 3(1): 15-20.

Vij, R., Vij, H. and Rao, N.N. 2011. Evaluation of collagen in connective tissue walls of Odontogenic cysts – A histochemical study. *J Oral Pathol Med.,*40: 257-262.

Zhang, J.Y., Dong, Q. and Li, T.J. 2011. Differences in collagen fibers in the capsule walls of parakeratinised and orthokeratinised odontogenic cysts. *Int J Oral Maxillofacial Surg.,* 240: 1296-300.

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