ORIGINAL RESEARCH PAPER

Pathology

ESTIMATION AND EVAULATION OF COLLAGEN FIBRES AS STROMAL COMPONMENT IN VARIOUS GRADES OF ORAL SQUAMOUS CELL CARCINOMA USING PICROSIRIUS RED STAIN UNDER POLARISED MICROSCOPE

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ABSTRACT

BACKGROUND: Oral squamous cell carcinoma (OSCC) is the 3rd most common form of malignancy in developed countries . The tumour is composed of neoplastic cells and the reactive stroma . Use of Picrosirius red (PSR) stain along with circular polarising microscope is helpful to detect stromal changes and the spread of malignancy and guides the treatment plan assess prognosis . The present study is aimed to analyse the various aspects of collagen fibres in relation with the different grades OSCC. **MATERIAL & METHODS:** This was cross sectional retrospective study done from 1st January 2015 and 31st December 2015 .30 patients of OSCC of various grades and 10 cases of normal mucosa were evaluated and data analysed statistically using sigma scan pro image analysis software. **OBSERVATION :** Among the thirty OSCC patients 19 were male and 11 were female, all within age group 47-60 years were analysed. There were 14 well differentiated (WDSCC),12 moderately differentiated (MDSCC) and 4 poorly differentiated (PDSCC) cases. When examined under polarising microscope majority (85.7%) of WDSCC and 41.67% of MDSCC showed reddish orange (RO) collagen fibres whereas in PDSCC (50%) showed green yellow (GY) collagen fibres which is statistically significant (P=0.02) .In WDSCC 57.14% and in MDSCC 50% had swirl(S) arrangement whereas in PDSCC 50% had cross hatchet (CH) and bundle arrangement .This is again statistically significant (P=0.031).While examining the orientation of collagen fibres in WDSCC 71.43% and in MDSCC 83.33% had parallel orientation whereas in PDSCC 75% showed non parallel orientation .This orientation is statistically significant (p.008). On comparing the nature of birefringency maximum cases of WDSCC (85.71%) and MDSCC (83.33%) revealed strong birefringency whereas in PDSCC 75% exhibited weak.(statistically significant p.001). **CONCLUSION :-** PSR stain under polarising microscope is an adjunct to the routine staining for studying stromal changes .The colour ,arrangement, orientation & birefringency changes reflect its gradual shift from WDSCC to PDSCC and may help as a diagnostic and prognostic tool in OSCC.

INTRODUCTION:-

Oral squamous cell carcinoma (OSCC) represents the third most common form of malignancy in developing countries while in developed countries it is the eighth most common form of cancer [1,2]. The tumour is composed of two basic components of which the neoplastic cells which constitute the parenchyma and the other component in the form of reactive stroma chiefly the connective tissue supporting the cell.[3] Extra cellular matrix (ECM) changes which indicate the propensity of tumour cells to infiltrate and metastasise can also be used as one of the prognostic indicators [4,5].Many markers are now available to study the tumour stroma interface and the ECM, but morphologic detection on Haematoxylin and Eosin (H and E) and special stain for connective tissue fibres is cost effective and can be used to advise the surgeon regarding the surgery .[6]

Picrosirius red (PSR) staining method in combination with polarising microscopy appear to be the most suitable method to visualise collagen fibres with high contrast.[7,8] While analysing the various grading systems proposed by many authors the Broder's grading system is found to be more relevant to the present study in the way that the various modes of invasion of malignant tissue into the underlying connective tissue zone is directly favoured by the response of the stromal changes .The present study aims to analyse the various aspects of collagen fibres in relation with different grades of OSCC using combination of picrosirius red stain and circularly polarized microscope and to know the specificity and distribution of collagen fibres in oral OSCC using a simple and reproducible histochemical method.

The present study was conducted to study and analyse the stromal collagen fibres in different grades of OSCC and observe their spatial arrangement using picrosirius red stain under circular polarising microscope and to find an observable stromal change with the progression of the disease. The present study also compared the pattern and distribution of collagen fibres in various grades of OSCC.

MATERIAL & METHOD:-

This cross sectional retrospective study was conducted between 1st January 2015 and 31st December 2015 in collaboration with the department of Oral Pathology and Microbiology of SCB Dental college and Hospital ,Cuttack with Department of Pathology in S.C.B Medical College, Cuttack. The study was approved by institutional Ethical Committee of S.C.B. Medical College and Hospital, Cuttack.

Sample collection :- In the present study total 30 cases of various grades of OSCC using Broder's criteria and 10 cases of normal mucosa were included In the present study.Samples were collected from alveolo -buccal complex and buccal mucosa. Paraffin embedded blocks of histologically diagnosed cases of OSCC of different grades(well differentiated, moderately differentiated and poorly differentiated) were retrieved from the archives of the department of oral pathology and microbiology of SCB Dental College and Hospital Cuttack, Odisha.

Normal oral mucosal samples were obtained from the pericoronal tissue of patients who had under gone surgery for impacted third molar. The tissue specimens were categorized as test group and control group. Only the specimens with adequate connective tissue thickness were taken for the study. All the cases selected for the study were selected as per the inclusion and exclusion criteria. Written informed consent was taken from the patients after they had been properly explained about the purpose of the same.

Inclusion Criteria: - Patients of age group of 30-80 years with incisional and excisional biopsies of conventional primary OSCC were taken into study.

Exclusion Criteria – Recurrent cases of OSCC, secondary or distant metastatic cases of OSCC and presences of any systemic or local collagen disorders were excluded from the study.

Study Method: Sections obtained for the study were first stained with Haematoxylin and Eosin using standard staining procedure and then graded according to Broder's criteria. After histopathological confirmation additional sections were further subjected to staining with picrosirius red stain and were observed under polarising microscope.

Analysis – The section were analysed using a research microscope (mode – LM -52-3525) with polarized illumination. All the sample were analysed by two observers to check inter observe variability and the data was computed, Kappa test was performed and it showed reliability between the two observers.

OBSERVATION :–
Thirty samples with OSCC were taken along with the control of 10 samples. The mean age of patients with well differentiated, moderately differentiated and poorly differentiated carcinoma were 47.35,58 and 60.75 years respectively.Table 1 depicts the comparison of polarising colors of collagen fibres observed in the three grades of OSCC. These was a significant difference in the polarising colors observed in the three grades of OSCC (P=0.02).

TABLE -1 COMPARISON OF POLARISING COLOURS OF COLLAGEN FIBRES OBSERVED IN THE THREE GRADES OF OSCC
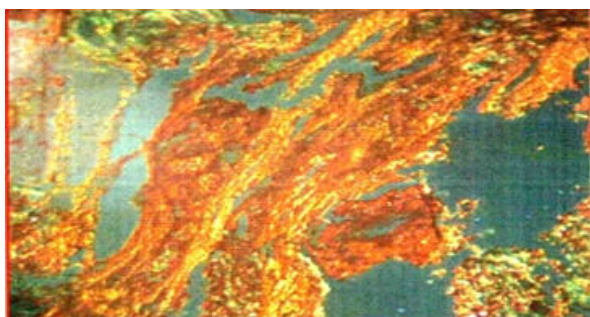
Polarising colours	Well differentiated carcinoma (n=14)	Moderately differentiated carcinoma (n=12)	Poorly differentiated carcinoma (n=4)	Chi square	P
GY n(%)	0(0)	2(16.67)	2(50)	10.85	0.02
YO n(%)	2(14.29)	5(41.67)	1(25)		
RO n(%)	12(85.71)	5(41.67)	1(25)		

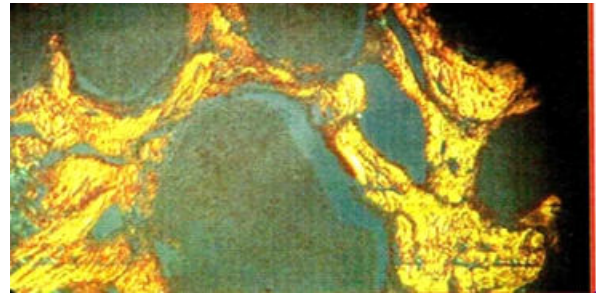GY- Greenish Yellow YO- Yellow Orange RO – Reddish Orange

IMAGES SHOWING DIFFERENT POLARISING COLORS OF COLLAGEN FIBRES IN DIFFERENT GRADES OF OSCC
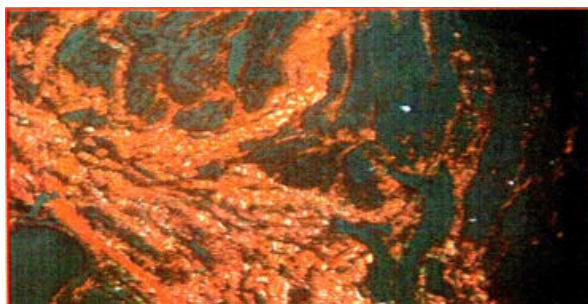
Image 1:Reddish Orange Collagen Fibre in WDSCC

Image 2: Yellow Orange to greenish yellow Collagen Fibre in MDSCC


Image 3: Greenish Yellow Collagen Fibre in PDSCC

Images 1, 2 and 3 depicts the different polarising colors of collagen fibres in different grades of OSCC.

Histopathological correlation of collagen fibres arrangement in three grades of OSCC were described in Table No 2 There was also significant difference between the arrangement of collagen fibres between three groups (P=0.031).
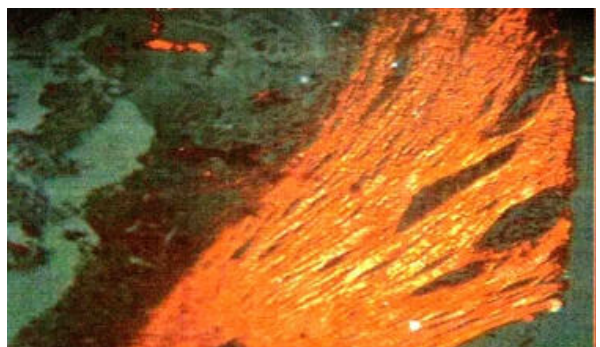
TABLE -2 HISTOPATHOLOGICAL CO-RELATION OF COLLAGEN FIBRES ARRANGEMENT IN THE THREE GRADES OF OSCC UNDER POLARISING MICROSCOPE.

Arrangement	Well differentiated carcinoma (n=14)	Moderately differentiated carcinoma (n=12)	Poorly differentiated carcinoma (n=4)	Chi square	P
Bundle n(%)	2(14.28)	2(16.67)	2(50)	4.74	0.031
Cross Hatchet n(%)	4(28.57)	4(33.33)	2(50)		
Swirl n(%)	8(57.14)	6(50)	0(0)		

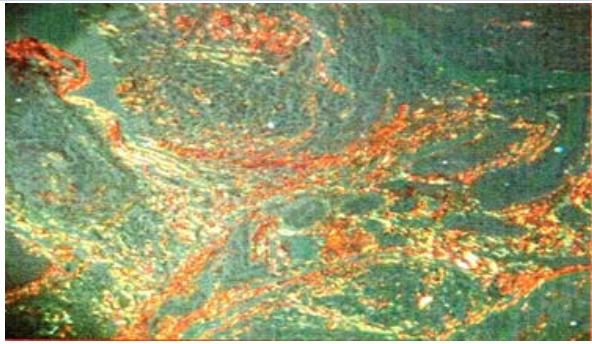**IMAGES SHOWING DIFFERENT TYPES OF ARRANGEMENT OF COLLAGEN FIBRE IN DIFFERENT GRADES OF OSCC**
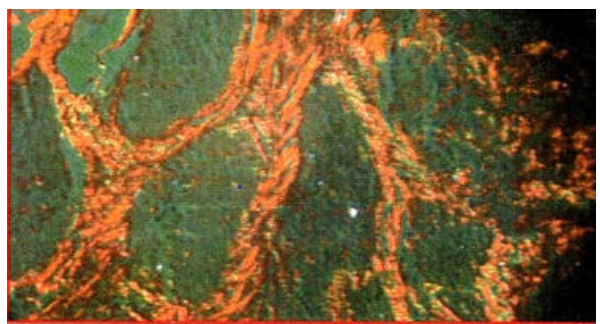
Image 4: Swirl Arangements of Collagen fibres

Image 5: Bundle Arangements of Collagen fibres

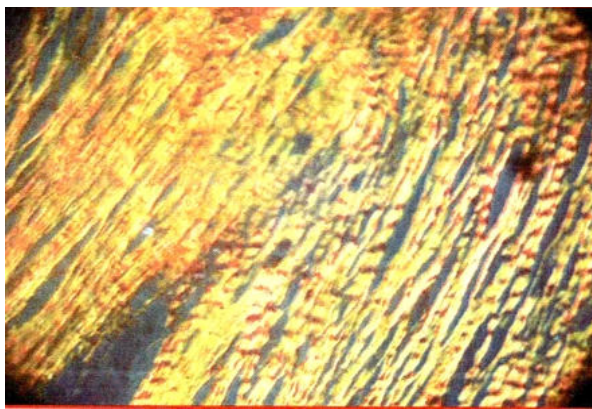**Image 6: Cross hatchet arrangements of collagen Fibres**

The image 4, 5 and 6 show the different types of arrangement of collagen fibres in different grades of OSCC.

The orientation of collagen fibres observed in the three grades of OSCC under polarising microscope was compared in Table No 3 There was a significant difference in the orientation of collagen fibres between these three groups(P=0.008).

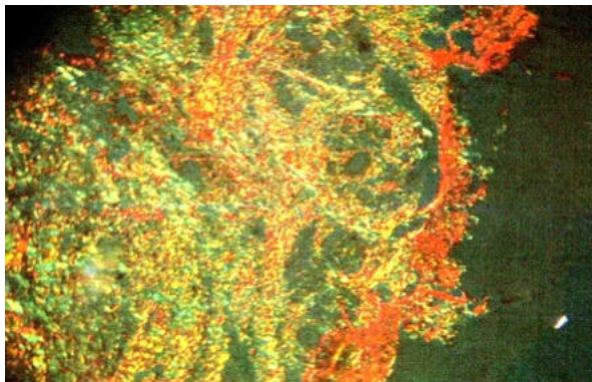**TABLE -3 COMPARISON OF THE ORIENTATION OF COLLAGEN FIBRES OBSERVED IN THE THREE GRADES OF OSCC UNDER POLARIZING MICROSCOPE.**

Orientation	Well differentiated carcinoma (n=14)	Moderately differentiated carcinoma (n=12)	Poorly differentiated carcinoma (n=4)	Chi square	P
Parallel n(%)	10(71.43)	10(83.33)	1.(25)	4.88	0.008
Non Parallel n(%)	4(28.57)	2(16.67)	3(75)		

IMAGES SHOWING ORIENTATION OF COLLAGEN FIBREOBSERVED IN DIFFERENT GRADES OF OSCC

**Image 7: Parallel arrangement of the collagen fibres**

Image 8: Non Parallel arrangement of the collagen fibres

Image 7 and 8 show the different types of orientation of collagen fibres in different grades of OSCC . Table 4 compares the nature of birefringency of collagen fibres in the three grades of OSCC under polarising microscope .It showed a significant difference in the nature of birefringency of collagen fibres in the three grades of OSCC (P=0.001).

TABLE -4:COMPARISON OF NATURE OF BIREFRINGENCY OF COLLAGEN FIBRES IN THE THREE GRADES OF OSCC UNDER POLARIZING MICROSCOPE

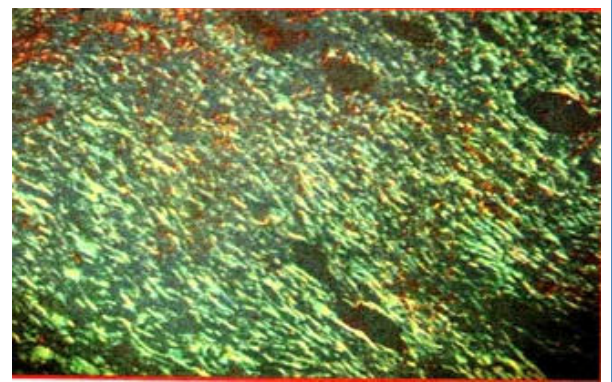| Nature of Birefringency	Well differentiated carcinoma (n=14)	Moderate differentiated Carcinoma (n=12)	Poorly differentiated Carcinoma (n=4)	Chi squre	P
Strong(%)	12(85.71)	10(83.33)	0(0)	17.53	0.001
Moderate D(%)	2(14.29)	1(8.33)	1(25)		
Weak n(%)	0(0)	1(8.33)	3(75)		

COMPARISON OF NATURE OF BIREFRINGENCY OF COLLAGEN FIBRES IN THE THREE GRADES OF OSCC UNDER POLARIZING MICROSCOPE
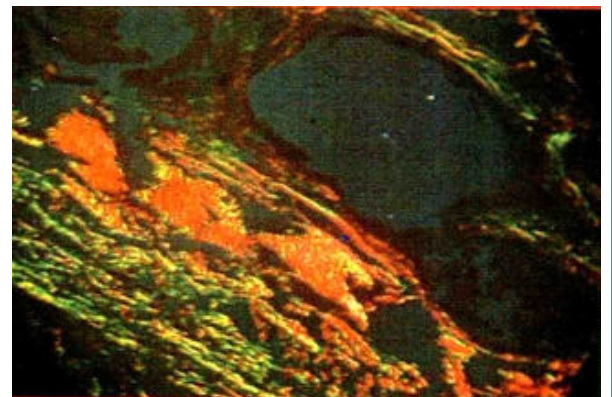
Image 9: Strong Birefringent Collagen Fibre
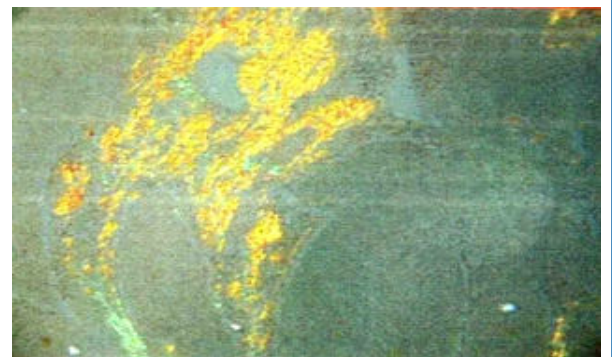
Image 10: Moderate Birefringent Collagen Fibre

Image 11 Weak Birefringent Collagen Fibre

Image 9,10 and 11 show the nature of birefringency of collagen fibres in different grades of OSCC.

DISCUSSION :-

The present study is aimed to study and analyse the stromal collagen fibres in different grades of OSCC and observe their spatial arrangement using picrosirius red staining (PSR) under circular polarizing microscope. 30 subjects were divided into three groups. The first group included 14 WDSCC cases whereas second group included 12 MDSCC cases and third group included 4 PDSCC cases. The mean age among WDSCC, MDSCC & PDSCC groups were 47.35,58 &60.75 years respectively. There was no significant difference in the mean age of different study groups. Present study shows 63.33% patients were male and 36.67%were female.

Thus study groups matched with sex distribution .Table 1 presented the distribution of study subjects as per the polarizing colours obtained by PSR staining and polarizing microscopy as observed in the three grades of oral squamous cell carcinoma. Out of 14 cases of WDSCC, majority (85.71%) showed reddish orange (RO) collagen fibres and yellowish orange (YO) in 14.29% .In MDSCC majority (41.67%) showed RO and YO fibres and few(16.67%) showed GY fibres .In PDSCC majority(50%) showed GY fibres, RO and YO fibres were observed in equal proportion of 25% each. Chi square test derived with p value 0.02(<0.05) confirmed such association to be statistically significant. This could be due to various growth factors and cytokines that cause proliferation of fibroblasts and ECM resulting in the formation of thick mature collagen.[9]

It may be noted that thick mature collagen normally exhibits RO colour[10]. Similar observation were also reported in the literature by Bhari baranesha Manjunatha et al in 2015 .[11] This indicated that in different grades of OSCC, there is gradual change in birefringence ranging from RO to YO and eventually to GY. There is a shift in polarising colours of collagen fibres in our study in MDSCC and PDSCC from YO to GY. This could be explained by the fact that as OSCC progresses, there is increased collagenolytic activity. The altered fibroblast phenotype produces altered collagen.[12] As the grade progresses from well to moderate to poor, there is a shift in collagen from type I(thick) to type III(thin) suggesting that as the carcinoma progresses the surrounding stroma co-evolves active state through continuous tumour stroma interaction.[13].

In the present study table 2 shows the relationship of the type of fibre arrangement in different grades of OSCC. Out of 14 cases of WDSCC, majority (57.14%) had swirl(S) arrangement of collagen fibres, few (14.28%) showed bundle (B) arrangement. Similarly, out of 12 MDSCC cases majority (50%) had swirl arrangement of collagen fibres, few (16.67%) had bundle arrangement. Out of 4 cases of PDSCC, majority (50%) had cross hatchet (CH) and bundle arrangement of collagen fibres. Swirl arrangement was absent. The correlation between the different types of arrangement of collagen fibres in the three grades of OSCC showed that the association was statistically significant (p value = 0.031). Similar result was also observed by Bhari Sharanesha Manjunatha, Aditi Agrawal in their study in 2015[11].

We presume that the intertwining of collagen fibres as seen in swirl arrangement tightens the fibres thereby delivering toughest resistance to tumour cells to invade. Whereas the collagen fibres in moderate and poorly differentiated OSCC are more disorganized due to increased stromal destruction that may enhance movement of tumour cells towards blood and lymphatic vessels[11] .Table 3 of our present study examined the orientation of the collagen fibres in relation to the tumour islands in the three grades of OSCC. Out of 14 cases of WDSCC, majority (71.43%) showed parallel orientation and 28.57% showed non parallel orientation .Out of 12 cases of MDSCC, majority(83.33%) showed parallel orientation of collagen fibres and 16.67% showed non parallel orientation. Out of 4 cases of PDSCC, majority(75%) showed non parallel orientation of collagen fibres and 25% showed parallel orientation.

The correlation showed that the association was statistically significant (p value 0.008). Similar result was also observed in the studies conducted by Bhari Sharanesha Manjunatha, Aditi Agrawal, in 2015[11]. On comparing the nature of birefringency (table 4) of the collagen fibres in the three grades of OSCC both the observers noted that in maximum patients there was strong birefringence of collagen fibres in well differentiated (85.71%) and moderately differentiated (83.33%) OSCC whereas in poorly differentiated carcinoma, majority(75%) exhibited weak birefringence .Chi square test was done and the association was found to be statistically significant with p value 0.001. This is also supported in the literature in the studies conducted by Bhari Sharanesha Manjunatha et al in 2015[11] .

CONCLUSION :

In the present study, observable collagenous changes were seen in progressive grades of OSCC. We have demonstrated that the combination of PSR with polarization microscopy is a powerful tool for its structural analysis. The observations of the present study indicate that picrosirius red (PSR) stain is an adjunct to the routine staining for studying stromal changes at the invading front of the tumour and this in turn aids in predicting tumour behaviour. The colour changes reflect its gradual shift from thicker mature fibres towards thinner immature fibres in stroma during tumour progression. In this study the significant variation in arrangement, orientation and nature of collagen (indicated by strength of birefringence), in different grades of OSCC proves increased disorganization of stroma with tumour progression which may be valuable as a diagnostic and prognostic indicator more so as a supplement to parenchymal changes. Thus, it should be supplemented with gold standard i.e., H and E and molecular markers on a larger sample including the tumour invasion front to extrapolate this knowledge. Further research with larger sample size is required in this direction.

LIMITATIONS OF THE STUDY:-

Although the PSR staining in combination with PM is a precise, effective and specific technique to study collagen, still as with all methods, it suffers from some limitations like few collagen fibres that appear crimped or wavy may appear dark irrespective of rotated microscope stage, resulting in underestimating and analysing their volume. Another limitation of the study is the green colour, despite its specificity for thin collagen fibre/ type III fibres may also appear in immature type I fibre, raising a lot of conundrums.

CONFLICT OF INTEREST : Nil

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