"ASSESSMENT OF COLLAGEN BIREFRINGENCE IN DIFFERENT GRADES OF ORAL SQUAMOUS CELL CARCINOMA USING PICROSIRIUS RED – POLARIZING MICROSCOPY AND COMPARISON WITH PSR – FAST GREEN STAIN"

DISSERTATION

Submitted to

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In the partial fulfilment of the requirements for the degree

of

MASTER OF DENTAL SURGERY

In

ORAL & MAXILLOFACIAL PATHOLOGY & ORAL MICROBIOLOGY

By

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Under the guidance of

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I hereby declare that this dissertation entitled **"ASSESSMENT OF COLLAGEN BIREFRINGENCE IN DIFFERENT GRADES OF ORAL SQUAMOUS CELL CARCINOMA USING PICROSIRIUS RED – POLARIZING MICROSCOPY AND COMPARISON WITH PSR – FAST GREEN STAIN"** is a bonafide and genuine work carried out by me under the guidance of **DR. JIJI GEORGE**, Professor & Head, and **DR. ANKITA SINGH,** Reader as co-guide in Department of Oral & Maxillofacial Pathology & Oral Microbiology, Babu Banarasi Das College of Dental Sciences, Babu Banarasi Das University, Lucknow, Uttar Pradesh.

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"Dedicated Solely To My Parents, Hod (guide) & Co-guide"

ACKNOWLEDGEMENT

ACKNOWLEDGEMENT

"DEDICATED SOLELY TO MY PARENTS, HOD (GUIDE) & CO-GUIDE"

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Dr. DEBA KUMAR DAS

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LIST OF ABBREVIATIONS

OSCC	ORAL SQUAMOUS CELL CARCINOMA
PSR	PICROSIRIUS RED
FG	FAST GREEN
ECM	EXTRACELLULAR MATRIX
WDSCC	WELL DIFFERENTIATED SQUAMOUS CELL CARCINOMA
MDSCC	MODERATELY DIFFERENTIATED SQUAMOUS CELL CARCINOMA
PDSCC	POORLY DIFFERENTIATED SQUAMOUS CELL CARCINOMA

ABSTRACT

ABSTRACT

Background and Objectives: Globally, oral cancer is the sixth most common type of cancer in India. Though Oral Squamous Cell Carcinoma is an epithelial malignancy, the invading tumor components are believed to influence the surrounding connective tissue. Stroma can aid in invasion by lysing the collagen or stop progression by increasing desmoplasia around the tumor cells. Hence, the present study was conducted to assess collagen in well/moderately/poorly differentiated Oral Squamous Cell Carcinoma using Picrosirius Red stain and PSR-Fast Green stain and comparison of both.

Materials and Methods: Study subjects included 20 samples of previously diagnosed Oral Squamous Cell Carcinoma and 10 samples of normal mucosa from healthy individuals as controls. Slides of previously diagnosed cases were retrieved from the archives & stained with Picrosirius Red, PSR-Fast Green and H&E and viewed under polarized light and bright field microscopy respectively. Morphometric analysis was done using Image J software.

Results: We observed thick, parallelly arranged, strongly birefringent dense collagen fibers showing predominantly Orange-Red polarisation in well differentiated Oral Squamous Cell Carcinoma while poorly differentiated Oral Squamous Cell Carcinoma revealed thin loosely arranged weakly birefringent haphazard collagen fibers with a green hue.

Conclusion: It can be concluded that collagen acts as a barrier and prevents the spread of tumour cells in the stroma. This property can be used to modulate the stromal composition which in turn can modify the epithelial tumour behavior.

INTRODUCTION

INTRODUCTION

Oral Squamous Cell Carcinoma is a rampant health crisis in the Indian subcontinent; estimated to account for 80% of all oral & maxillofacial malignancies. It is primarily an epithelial neoplasm; but much emphasis and research has been done on the role of stromal components in the tumor behavior. Collagen is a major component of intervening and surrounding stroma in Oral Squamous Cell Carcinoma. The role of collagen can be protective or otherwise. Though fiber bundles can be visualized using H/E stain, differentiation of collagen fibers from elastin and reticulin is possible with special stains like Van Gieson and other trichrome techniques. Picrosirius Red (PSR) is a prominent choice of special stain for collagen, which can be visualized under polarizing microscopy. Picrosirius Red can be used under brightfield microscopy as well but may not be specific for collagen. Hence we combined Picrosirius Red with Fast Green to evaluate the differentiation potential of the combination stain to easily separate & visualize collagen from non-collagenous components. Though numerous studies have been documented in literature, comparing PSR with other trichrome stains, not much has been done towards combining picrosirius red with other stains.

Therefore, we designed this study to use PSR solely as well as in combination with Fast Green to compare their staining efficacy to detect and analyze collagen fibers in the stroma of Oral Squamous Cell Carcinoma.

AIM AND OBJECTIVES

AIM & OBJECTIVES

AIM:

To assess collagen in different grades of Oral Squamous Cell Carcinoma using Picrosirius Red stain and PSR-Fast Green stain.

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OBJECTIVES:

1. Assess and compare Hue in all grades of Oral Squamous Cell Carcinoma stained with Picrosirius Red using Polarized light microscopy.
2. Assess and compare staining of collagenous and non – collagenous components in Picrosirius Red and PSR – Fast Green stained slides in all grades of Oral Squamous Cell Carcinoma.
3. Assess and compare birefringence in Picrosirius Red and PSR – Fast Green stained slides in all grades of Oral Squamous Cell Carcinoma.
4. Assess the thickness (thick/thin), orientation (haphazard/parallel) and arrangement (dense/loose) of fibers in all grades of Oral Squamous Cell Carcinoma using Image J.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

ORAL SQUAMOUS CELL CARCINOMA: -

Oral squamous cell carcinoma (OSCC), commonly occurring head and neck cancer, has high prevalence in certain parts of the world, and is associated with a high mortality rate[1].

Oral cancer holds the eighth position in cancer incidence worldwide, with epidemiologic variations in different geographic regions[2].

Recent data indicate that the incidence of neoplastic head and neck lesions is high with squamous cell carcinoma ranked sixth worldwide. In the United States more than 21,500 cases of oral carcinoma are diagnosed annually, because of which more than 6,000 Americans die each year[3].

World Health Organization (WHO) published its data in 2008, which states that as many as 7.6 million people worldwide die due to cancer, with 70% of the cases of cancer deaths occur in developing countries while, only 30% are successfully treated[4].

In south-central Asia, it is the third most common malignancy[2].

It has been reported in recent study that 14.1 million new cancer cases and 8.2 million cancer deaths happened around the world in 2012. Amongst which, 3,00, 400 new cases cancer had been reported in lip and oral cavity, with1,45, 400 deaths, which accounts for more than 2% of new cases and 1.7% cases of death in the world respectively[5].

India contributes to almost one-third of the total burden and the second country having the highest number of oral cancer cases. Oral squamous cell carcinoma (OSCC) contributes remarkably i.e. 84-97% to oral cancer with potentially malignant disorders, recognized as a detectable pre-clinical phase of oral cancer[6].

Despite the advances of therapeutic approaches, percentages of morbidity and mortality of OSCC have not improved significantly during the last 30 years. Percentages of morbidity and mortality in males are 6.6/100,000 and 3.1/100,000 respectively, while in females the same percentages are 2.9/100,000 and 1.4/100,000[7].

Oral cavity malignancies arises most commonly on buccal mucosa, tongue, lips, and floor of the mouth, gingiva and hard palate. Histologically, there is no significant difference in oral malignancies at various sites of the oral cavity. But, they show variability in behaviour and prognosis. Most of the patients are asymptomatic only with vague symptoms. The symptoms include difficulty in swallowing, chewing, opening the mouth, weight loss, oral bleeding and neck swelling. Patients presenting with red or white plaques are closely observed especially with the habit of tobacco and alcohol consumption. The advanced disease usually presents with the proliferative growth or rarely with subcutaneous nodules and orocutaneous fistula[8].

Squamous cell carcinoma of the lip constitutes about 24 % to 30% of the oral cancers and among head and neck malignancies, it is 12%. 85% to 98% of lip cancers are seen in the lower lip with male predominance[9].

Early lesions usually present as focal white, or erythematous lesion, while advanced lesions present more commonly as an ulcer, but, some advanced lesions are present

as exophytic, infiltrating lesions. Palpable induration surrounding the lesion forms the hallmark feature in all forms of tumor presentation[10,11,12].

In India, squamous cell carcinoma of buccal mucosa constitutes around 44% of all oral squamous cell carcinoma. Males are more commonly involved. Most of the cases, were in seventh to eighth decades[13,14].

Early lesions are white plaque, red plaque, red macule, or verrucous hyperplasia, while advanced tumors appear as a fungating mass or as an ulcerative infiltrative cancer. The involvement of lamina propria, buccinator muscle and buccal fat forms the main hall mark of the tumor[14,15].

Floor of the mouth and the tongue are the most common sites of oral SCC. They constitute about 60% of oral SCC. Smoking, Tobacco use and Excessive alcohol abuse are the main etiological factors. Usually, it occurs in elderly patients (6th to 7th decade) but at present, its incidence is found even in younger age group. (Less than 40 years)[16,17].

Most advanced tumors are symptomatic, but the most common presenting symptoms are feeling of discomfort, pain, limitation of movement, slurred or difficulty in speech, excessive salivation, weight loss and hemorrhage. Involvement of the base of tongue may present as dysphagia and referred otalgia, also, lymph node metastasis are commonly seen. The nodes more commonly involved are the submandibular and the upper jugular nodes. This nodal involvement is found to have a positive correlation with the tumor size, which forms the independent significant predictor for the lymph node metastasis. Distant metastasis is rare, occurring in about 10% of cases, with the lungs, liver and bone being the most common sites[18,19].

Gingival SCC ranks third among the oral SCC occupying 4-25%. The important risk factors are tobacco usage, alcohol consumption, snuff dipping and poor oral hygiene[20].

Hard palate forms the rarest site for the development of SCC, but, it is seen in those areas with Chutta smoking. The peak age group is 60 and 70 years. The disease is more common among males and patients usually presents with ulcer or an exophytic growth measuring less than 4 cm in diameter. Lymph node metastasis more commonly seen to spread to submandibular and sub digastric lymph nodes[21,22].

Upon early diagnosis, timely and proper treatment can be initiated that may improve the survival rate up to 90%. With advancements in science and technology, numerous novel techniques have been developed that have advantages as compared to the currently practiced conventional diagnostic methodologies[6].

TUMOR PROGRESSION

The tumor progression in OSCC is accompanied by degradation of the basement membrane and extracellular matrix which occurs during local invasion, angiogenesis, vascular and lymphatic invasion[23].

The morphological signs of cancer- associated stromal alterations are desmoplasia, angiogenesis and inflammatory cell infiltration[24].

Stroma can aid in invasion by lysing the collagen or can stop progression by increasing desmoplasia around the tumor cells. Collagen can either mount a desmoplastic response to a tumor & cause excess deposition of collagen around it or degrade and decrease collagen synthesis allowing invasion of tumor cells through the

stroma. Degradation of the extracellular matrix is dependent on specific interactions between tumor and host cells[25].

Oral squamous cell carcinoma showed local invasion of the underlying connective tissue in forms of islets and cords of epithelial cells. Interaction between tumor cells and ECM components is essential for the tumor growth and onset of distant spread and onset of metastatic activity[26].

Hallmark of carcinoma is the neoplastic cell migration and invasion. During the transformation from dysplasia to carcinoma, hypoxia arises which causes genetic instability and accelerate angiogenesis thus making the stroma edematous and unstable and as carcinoma progresses, neoplastic cell transform collagen mainly by the production of Carcinoma Associated Fibroblasts (CAFs) and increase collagenolytic enzyme activity. This altered fibroblast phenotype causes production of altered collagen. Also, by increased formation of collagenases, the invading neoplastic cell dissolve the collagen eventually which leads to a disarranged stroma[27].

Documented literature reveals that polarizing colors of collagen fibers show a gradual change from reddish orange to greenish yellow from well to poorly differentiated squamous cell carcinoma, thus indicating that as the tumor progresses, there is a change from the mature collagen to an immature form[28].

The extracellular matrix surrounding tumor cells undergoes changes along with tumor progression. Extensive changes of the normal extracellular matrix into the

matrix of the tumor consists of degradation of matrix components and/or new synthesis of matrix components that are not found in normal tissue[29].

The ECM macromolecules alter cellular events such as adhesion, migration, proliferation and differentiation. Tumor cells cause proteolysis of ECM, which modifies its structure and facilitates migration of the tumor cell[30].

The production of extracellular matrix components is increased in the stroma surrounding the tumor cell. Tumor stroma has an abundant amount of immune cells, endothelin, and fibroblasts. Due to the effects of mass suppression by tumor cells, fibroblasts in the stroma undergo differentiation and obtain the phenotype resembling myofibroblast. Fibroblasts which have this myofibroblast phenotype produce reactive stroma which has different characteristics from stroma in normal cells. Stromal tumor has a number of ED-A fibronectin, tenascin-C, and type I collagen[31].

Increased production of extracellular matrix components is associated with poor prognosis in ovarian carcinoma[32]. There is an increase of collagen type III intensity and decrease in type I collagen in benign ovarian tumors. The production of collagen in benign ovarian tumors is the result of the fibroblasts. Although in malignant ovarian tumors the synthesis of collagen increases, the total collagen decreases as compared to benign tumors[7].This change is because in the malignant tumor there is degradation of extracellular matrix components in the stroma, because of the presence of matrix metalloproteinase enzymes[33].

Changes in the structure of collagen that induce the interaction between tumor cells and stroma mark the initiation of the process of EMT[34].

Degradation and redeposition of collagen in the stroma regulate the microenvironment around the tumor. Collagen is a physical barrier against invasion of tumor cells, but it is also known in inducing infiltration, angiogenesis, invasion, and migration of tumor cells[35].

In cancer, there is a persistent secretion of collagen-destroying enzymes (collagenases, proteinases) by cancer cells causing the destruction of the surrounding collagen[5].So, the surrounding collagen fibers are dissolved or becomes immature, facilitating tumor growth and metastasis[36].

In this context it becomes imperative to understand the role of collagen in tumour progression.

Collagen

Collagen is a major component of intervening and surrounding stroma in OSCC's. The role of collagen can be protective or otherwise. Though fiber bundles can be visualized using H/E stain, differentiation of collagen fibers from elastin and reticulin is possible with special stains.

The mechanical quality of ECM is mainly dependent on its collagenous content and presence of collagen is considered a main barrier to be cleared away during invasion, thus making room for infiltrating cell mass[37].

The extra- cellular matrix mainly consists of type I collagen, which is about 90%, and type III collagen, which is 8–10%. Electron microscopic studies have shown that type I collagen fibres are coarse and are composed of closely packed thick fibrils,

whereas type III collagen forms thin fibres and are composed of loosely disposed thin fibrils[38].

Thin fibres show GY polarization which increases with dedifferentiation of OSCC and thick fibres with YO polarization decreased with dedifferentiation of SCC. The increase in thin fibres (type3) and decrease in thick fibres (type1) with dedifferentiation of OSCC could be due to the initial fibroproliferative response and in later stages there will be abnormal collagen production and defective maturation which may promote the neoplastic growth[39].

Sharf et al[40]in their study on physical aggregation of the collagen fibers revealed a color profile of orange to red, which corresponded to well-packed fibers and green to greenish yellow corresponded poorly packed fibers.

Collagen around tumor islands could be of stromal origin, and play a role in walling off the invading tumor cells[41].

Gangana et al[42] suggested that change in polarization colors of collagen fibers may be indicative of neoplastic transformation.

Polarizing colors of collagen fibers could be due to various growth factors and cytokines that causes proliferation of the fibroblasts and ECM resulting in the formation of thick mature collagen fibers. As the collagen matures, the change in proteoglycans content of the fiber causes dehydration of these fibers thereby,

increasing the diameter of the collagen fibers. Therefore,due to tight packing of collagen, there could be difference in polarizing colors[43].

Dayan et al[44] revealed that packing of the collagen fibers play an important role in pattern of polarization andcolours of PSR stained collagen. Tightly packed and well aligned collagen fibers showed polarization colours of longer wavelengths (reddish orange).

Picrosirius Red

Picric acid in 1771 was first used as a synthetic dye for silk. 'Picric acid' is a trinitro-aromatic compound & derived from Greek word "pikros" which means "bitter"[45].

In 1889, Ira Van Giesoncombined picric acid and acid fuchsin, which was a successful histological technique despite the fact that the red color of the stained tissue vanished within a short time span. In 1964, Picrosirius Red stain was first used by Sweat et al by combining Sirius red F3BA (also known as F3B or Direct Red 80) with picric acid[46].

Picrosirius red is a prominent choice of special stain for collagen, which can be visualized under polarising microscopy. Though numerous studies have been documented in literature, comparing PSR with other trichrome stains, not much has been done towards combining picrosirius red with other stains. Combining Picrosirius red with Fast green can be used to increase the differentiation of collagen from other constituents of the connective tissue.

Traditionally, stains like Van Gieson and other trichome stains were used to demonstrate collagen fibers in tissue sections, but they lacked precise selectivity and failed to reveal very thin collagen fibers. Sirius Red, an elongated dye molecule, reacts with collagen with high specificity and promotes enhancement of the normal birefringence of collagen under polarizing microscope[47].

As collagen is a basic amino acid and has a strong affinity for acidic dyes, sirus red being an elongated dye molecule responds with the collagen and increases its birefringence[48].

Sirius red, which is an elongated dye molecule reacts with collagen and amplifies its normal birefringence. It's because many dye molecules are parallely aligned with the long axis of each collagen molecule. It has also been stated that this dye binds to collagen through a strong interaction of its acid sulfonic groups to collagen molecules[44].

Picrosirius red stain has capability to detect thin collagen fibers which are not possible with routine staining procedure, so it helps to differentiate between mature and immature collagen fibers[49].

Picrosirius red stain is an adjunct to the routine staining for studying stromal changes at the invading front of the tumour islands and this, in turn, aids in predicting tumour behaviour[28].

It is well known that the collagen viewed under polarized light is birefringent. **Constantine et al**[47] revealed that the intensity of the birefringence after Sirius red is far higher than seen either in unstained sections or after any other procedures.

Also, Sirius red does not impart birefringence to otherwise non - birefringent structures, which helps to distinguish collagen from other substances that are also stained by Sirius red.

Krishna Singh Arora et al[50]in their study revealed that half of the cases of oral squamous cell carcinoma showed green-yellow birefringence, suggesting that oral malignancy indeed is associated with a breakdown of matrix.

Venigella and Charu[28]in their study reported that in well differentiated squamous cell carcinoma and moderately differentiated squamous cell carcinoma, distinct deposits of collagen showed reddish orange to yellowish orange birefringence, which was mainly concentrated around the tumor islands. Whereas, moderately differentiated and poorly differentiated squamous cell carcinoma in the same study showed a gradual change in polarizing colors from yellowish orange to greenish yellow, particularly in the immediate vicinity of the invading tumor islands. The transformation of lesions from a pre-neoplastic to cancerous state is associated with an increase in collagenolytic activity.

According to **Montes et al**[38] color change can be attributed to the carcinogenic events, action of MMP's, pathological breakdown of the matrix by tumor cells, abortive stroma, thereby promoting the progression of tumor. They reported that

collagen profiling can be used effectively to correlate collagen qualitatively to tumor progression and clinical behavior of OSCC. Thus, the color changes seen are clear indicators of stromal tissue changes developing around the tumor cells, which can be related to neoplastic events taking place during tumorigenesis.

Ashalata G et al[51] in their study illustrated the parallel orientation of collagen fibers with respect to epithelium and colour changes observed in OSCC. The disparity in colour pattern of collagen fibers might be due to assorted growth factors and cytokines causing fibroblast proliferation and extracellular matrix which results in the arrangement of immature collagen.

Since collagen is converted from mature to immature, the modification in proteoglycan content of fibers could be the reason of dehydration of fibers thereby decreasing the diameter of the collagen fibers[52].

Priyanka Kardam et al[53] in their study showed a change in colors of collagen was observed from well to poorly differentiated SCC, with thin collagen fibers predominantly green yellow but thick fibers with variety of colours. As OSCC grade progressed, collagen fibers were loosely packed haphazardly arranged.

Pillai Arun Gopinathanet al[39] in their study revealed polarization colours of thick fibres were YO 79.8% in WDSCC and 55.7% in MDSCC mainly around the tumour islands, and that could be due to deposition of collagen fibres in the form of thick bands and closely packed fibrils. The change in polarization colours of thick fibres in

MDSCC and PDSCC showed a gradual change in birefringence from YO to GY around the tumour islands and that could be due to loosely packed fibres which might be composed of procollagens, intermediate or pathological collagen rather than normally tightly packed fibres. The change in the birefringence of thick fibres, could be because of the adjacent tumour cells which secrete enzymes such as collagenases or MMPs, disorganized stroma, and uninhibited proliferation of dedifferentiated tumour cells with secretion of their abnormal matrix. Similar changes were observed in polarization colours of collagen fibers in the different grades of OSCC carried out by **Aparna and Charu**[28].

PSR stain studies are usually done under linear polarized light. The disadvantage of using linear polarized light is that PSR-stained fibers will appear dark if they are aligned parallel to the transmission axis of either of the two linearly polarizing filters but this can be overcome by using rotating microscope stage which will change the orientation of the tissue section with respect to the transmission axes. Crimped or wavy collagen will appear dark irrespective of rotated microscope stage. Thus, the total collagen content especially in tissue containing large amounts of wavy fibers may be underestimated. Here, fiber hue does not permit identification of collagen fiber type. Type III fibers are usually thinner than type I fibers but the green colour does not necessarily signify type III and can also represent either an immature type I or sectioning artifact smeared of thick type I fiber. Also, materials such as keratin and fibrin are weakly birefringent which is almost similar to thinnest collagen fibers, thus complicating the analysis[38].

FAST GREEN

Fast Green dye is used for staining connective tissues and as a counter stain in combination techniques. It is a water soluble stain, belongs to triarylmethane dyes and is widely used in trichrome techniques. It can also be used as a substitute for Light Green SF dye in Papanicolou staining and also for histochemical staining.

PSR WITH FAST GREEN[54].

PSR- FG staining allows to highlight well defined red stained collagen and to obtain good quantitative results by morphometric image analysis.

PSR specifically binds the helical structure of fibrillar collagens whereas Fast Green binds to non-collagenous proteins. PSR absorption is at 540nm and FG is at 605 nm respectively. Collagen even in thin fibers can be detected against the green stained non collagenous proteins.

According to **-Lopez-De Leon A et al**[55] Sirius Red/Fast Green technique allowed to achieve a better estimation of collagen fibers in formalin-fixed paraffin-embedded sections, due to their red-staining against background of green-stained non-collagen proteins.

According to**, Cristina Segnani et al**[54] collagen fibers stained by Sirius Red alone appeared as red areas surrounded by a lot of reddish shades. The study also revealed that collagen fibers which stood against the green-stained non-collagen components, could be clearly appreciated even in thinner networks. In Sirius Red/Fast Green technique, since the fast Green dye, selectively stains non-collagen tissue components and gives rise to a useful color contrast and visualization of red-stained

collagen fibers, as well as an optimal threshold for counting the positive pixels, this method is used to obtain the best quantitative estimation of collagen content by image analysis. Consistently with this view, the Sirius Red/Fast Green staining has been widely used. Histochemical staining carried out by Sirius Red combined with Fast Green represents an excellent method for standing out collagen fibers, being a technique more sensitive than Hand E or Sirius Red alone in terms of morphological and quantitative evaluations.

POLARISING MICROSCOPY

Light can be described as electromagnetic vibration, where natural light vibrates in many directions. Polarised light has a property of uni-directional vibration. When natural light is passed through a polariser, it allows vibrations of only one direction to pass. Polarisers are crystals capable of producing plane polarised light. This property is called as birefringence. In polarizing microscopy, two polarisers are used; one at the back focal plane of the objective and the other at the back focal plane of the condenser. Rotation of the polariser at 45 degrees, will result in alternate appearance and disappearance of the image under a dark and bright backgrounds respectively. Collagen and quartz show positive birefringence[56].

Under polarizing microscopy, Collagen fibers can be grouped as type 1 (thick, 1.6-2.4 micrometer) and type 3 (thin, <0.8micrometer) with YO and GY polarization colors respectively. GY colors of both thin and thick fibers suggest that collagen is loosely packed. Tightly packed collagen shows a YO color[57].

Examination of collagen fibres by PSR helps to differentiate procollagens, and intermediate and pathological collagen fibres[58].

MATERIALS AND METHODS

MATERIALS AND METHODS

This retrospective study was conducted in the Department of Oral and Maxillofacial Pathology and Oral Microbiology, Babu Banarasi Das College of Dental Sciences, BBD University, Lucknow.

Study samples: We included 20 samples of previously diagnosed OSCC of all grades and 10 samples of normal oral mucosa from healthy individuals as controls. Paraffin embedded tissue blocks of these cases were retrieved from the archives of Department of Oral and Maxillofacial Pathology and Oral Microbiology, Babu Banarasi Das College of Dental Sciences, BBD University, Lucknow.

Eligibility Criteria:

Inclusion criteria:

- Histopathologically diagnosed cases of OSCC

Exclusion Criteria:

- Superficial biopsies without stromal depth
- Micro-invasive carcinoma
- Cases showing distant metastasis
- Recurrent OSCCs
- Patients undergoing chemotherapy or radiotherapy
- Patients with presence of any systemic illness

3 sections each of four micrometer thick sections were taken from each block and stained for H/E, Picro Sirius Red and PSR- Fast green respectively.

Sampling Method: Random Sampling

Materials and Equipments:

Reagents:

1. Coplin jars.
2. 2% (w/v) paraformaldehyde solution in phosphate buffer saline (PBS).
3. For formalin-fixed paraffin-embedded (FFPE) tissue sections: (a) Xylene or equivalent, (b) Absolute ethanol, (c) Deionized or distilled water (diH_2O).
4. Sirius red working solution: prepare 1 l of saturated picric acid (i.e., 1.3% (w/v) picric acid solution in diH_2O; also called 2,4,6-trinitrophenol), add 1 mg Sirius red F3B (also called Direct red 80, color index: C.I. 35782), and stir. The final concentration of Sirius red is 0.1% (w/v). Store at room temperature for several months.
5. Wash solution: add 5 ml glacial acetic acid (i.e., ~18 M) to 995 ml diH_2O. Store at room temperature.
6. Rehydration station: place eight Coplin jars containing xylene (2 jars), 100% ethanol (2 jars), 95% ethanol (1 jar), 90% etha- nol (1 jar), 70% ethanol (1 jar), and diH_2O (1 jar) in a fume hood.
7. Organic mounting medium such as Permount® or Cytoseal®, Pasteur pipettes with latex bulbs and coverslips

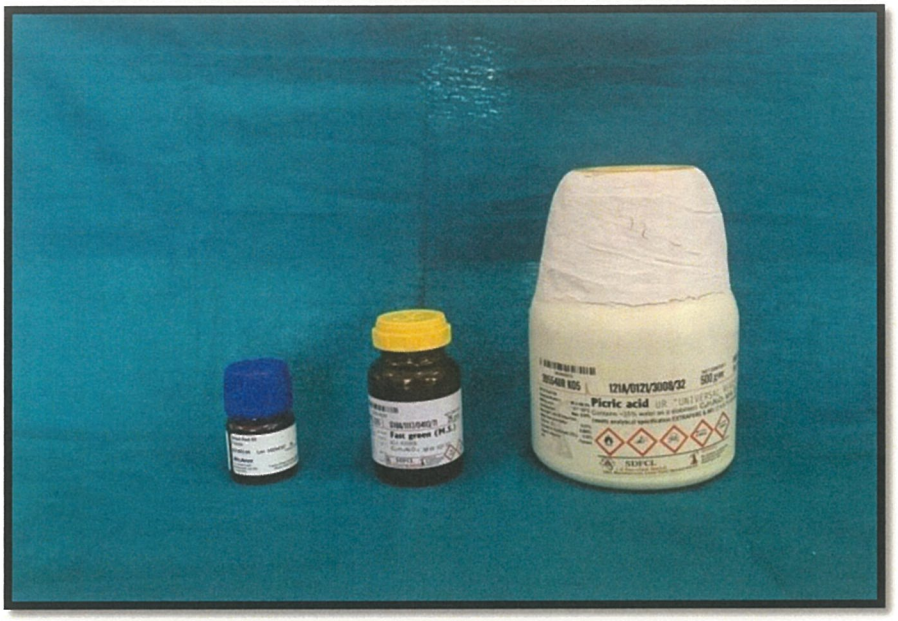

Fig 1: PSR and Fast Green Stain

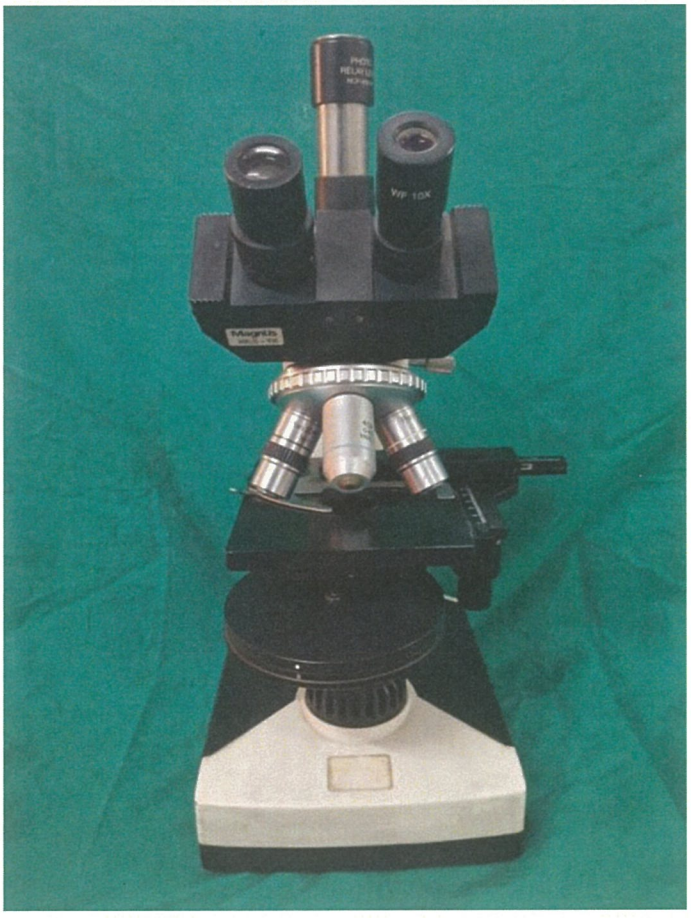

Fig 2: Polarising Microscope Olympus (MicronOptik model KG – 6 POL)

Methodology:

Methodology for H/E staining[56]:

1. Dewax sections, rehydrate through descending grades of alcohol to water.
2. Remove fixation pigments if necessary.
3. Stain in an alum hematoxylin of choice for a suitable time.
4. Wash well in running tap water until sections 'blue' for 5 minutes or less.
5. Differentiate in 1% acid alcohol (1% HCl in 70% alcohol) for 5–10 seconds.
6. Wash well in tap water until sections are again 'blue' (10–15 minutes), or
7. Blue by dipping in an alkaline solution (e.g. ammonia water), followed by a 5-minute tap water wash.
8. Stain in 1% eosin Y for 10 minutes.
9. Wash in running tap water for 1–5 minutes.
10. Dehydrate through alcohols, clear, and mount.

Methodology for Picro Sirius Red staining[59]:

1. Dewax FFPE tissue sections in three rinses of xylene (ten dips or 2–3 min incubation each), three rinses of absolute ethanol (ten dips or 2–3 min incubation each), and three rinses in diH_2O.
2. Stain in Sirius red working solution for 1 h.
3. Remove excess staining by washing twice with wash solution (ten dips or 2–3 min each).
4. Dehydrate slides by immersing them through a series of increasing alcohol concentrations: Transfer slides sequentially to diH_2O, 70%, 90%, 95%, and 100% (v/v) ethanol (ten dips or 2–3 min incubation each). Rinse slides to a second 100% ethanol solution to ensure that all water is removed.

5. Replace ethanol with xylene by immersing slides in two successive xylene solutions.
6. Mount slides with organic mounting medium and coverslips.
7. Observe stained tissue with polarized light microscope.

Methodology for Picro Sirius red-Fast green staining[54]:

Samples were incubated in 0.04% Fast Green for 15 min, washed with distilled water and then incubated in 0.1% Fast Green and 0.04% Sirius Red in saturated picric acid for 30 min. They were then dehydrated and mounted with DPX Mounting. Collagen fibers were differentiated as red, while the non-collagen proteins stained green.

Scoring criteria:

To eliminate subjective bias, the slides were evaluated by two observers independently using a Polarizing microscope, where 05 high power fields from each sample were randomly selected and visualized.

The interpretation and inter group comparison of polarizing hues of PSR stained collagen fibers was done under polarized light microscopy.

After Picrosirius staining, nature/categories of collagen fibers based on color were noted in each case of OSCC according method proposed by **Venigella and Charu[28]**:

Category 1: Reddish, Reddish Orange

Category 2: Yellowish, Orange, Yellowish, Green

Category 3: Greenish Yellow, Greenish

The hues gave a qualitative interpretation of fibers.

For quantitative inter group comparison, morphometric Image analysis was carried out with Image J software; and collagen fibers were grouped into thick and thin collagen fibers. Collagen fibers which measured 2micrometer- 10 micrometer were considered as thick fibers and those measuring between 0.5micrometer- 1.5 micrometer were considered thin fibers[60].

Collagen is a naturally birefringent substance. We assessed intergroup differences of this property under polarized light by categorizing the groups to cases showing weak birefringence or strong birefringence.

The pattern and orientation of collagen fibers were also assessed and compared between the different grades of OSCC. Tissues were graded as fibers which predominantly showed parallel orientation or with haphazard orientation.

The arrangement of fibers were categorized as loosely arranged or densely arranged.

Then, collagen fibers were also analyzed and compared for specific threshold of different colors using PSR- FG staining (e.g pink/red for collagen fibers against background of green stained non-collagen proteins)[54].

The obtained findings from above parameters were subjected to statistical analysis.

OBSERVATIONS AND RESULTS

OBSERVATIONS AND RESULTS

Table 1: Intergroup comparison of Hue in PSR staining

			Hue PSR			Total
			Orange Red	Yellow orange	Green yellow	
Type of SCC	Well differentiated	N	20	0	0	20
		%	100.0%	0.0%	0.0%	100.0%
	Moderately differentiated	N	4	16	0	20
		%	20.0%	80.0%	0.0%	100.0%
	Poorly differentiated	N	0	0	20	20
		%	0.0%	0.0%	100.0%	100.0%
Total		N	24	16	20	60
		%	40.0%	26.7%	33.3%	100.0%
P value						<0.001, S

Chi square test

Intergroup comparison of Hue in PSR staining was done using chi square test. All the specimens of well differentiated OSCC showed Orange red hue in PSR staining, while all the specimens of poorly differentiated OSCC showed green yellow hue in PSR staining. Majority specimens of moderately differentiated OSCC showed yellow orange hue. These differences were found to be statistically significant.

GRAPH 1: Intergroup comparison of Hue in PSR staining

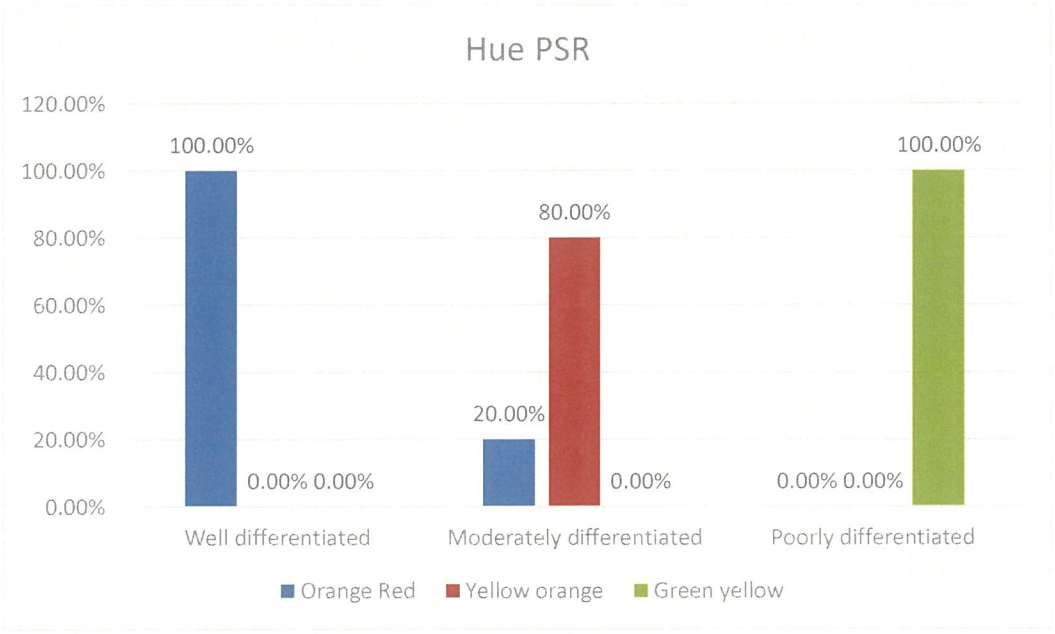

TABLE 2: INTERGROUP COMPARISON OF BIREFRINGENCE IN PSR STAINING

			Birefringence PSR			Total
			Weak	Strong	Stronger	
Type of SCC	Well differentiated	N	0	8	12	20
		%	0.0%	40.0%	60.0%	100.0%
	Moderately differentiated	N	12	8	0	20
		%	60.0%	40.0%	0.0%	100.0%
	Poorly differentiated	N	20	0	0	20
		%	100.0%	0.0%	0.0%	100.0%
Total		N	32	16	12	60
		%	53.3%	26.7%	20.0%	100.0%
P value						<0.001, S

Chi square test

Intergroup comparison of Birefringence in PSR staining was done using chi square test. Majority specimens of well differentiated OSCC showed Stronger birefringence in PSR staining, while all the specimens of poorly differentiated OSCC showed Weak birefringence in PSR staining. Majority specimens of moderately differentiated OSCC also showed Weak birefringence in PSR staining. These differences were found to be statistically significant.

Graph 2: INTERGROUP COMPARISON OF BIREFRINGENCE IN PSR STAINING

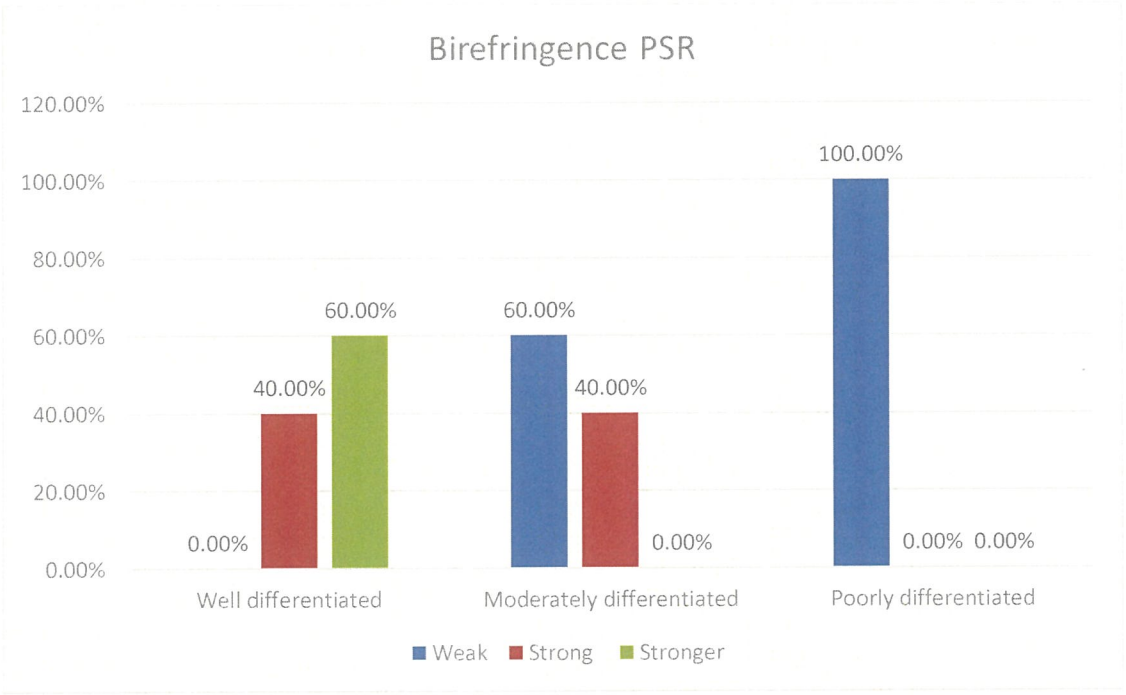

Table 3: Intergroup comparison of Differential staining intensity with PSR-FG stain

			Differential staining intensity PSR-FG				Total
			Very weak	Weak	Strong	Stronger	
Type of SCC	Well differentiated	n	0	12	0	8	20
		%	0.0%	60.0%	0.0%	40.0%	100.0%
	Moderately differentiated	n	4	12	4	0	20
		%	20.0%	60.0%	20.0%	0.0%	100.0%
	Poorly differentiated	n	0	12	0	8	20
		%	0.0%	60.0%	0.0%	40.0%	100.0%
Total		n	4	36	4	16	60
		%	6.7%	60.0%	6.7%	26.7%	100.0%
P value							0.001, S

Chi square test

Intergroup comparison of differential staining intensity with PSR-FG stain was done using chi square test. Majority specimens of well differentiated, moderately differentiated & poorly differentiated OSCC showed Weak differential staining intensity with PSR-FG stain. Remaining specimens of well- & poorly-differentiated OSCC, showed Stronger differential staining intensity, while remaining specimens of moderately differentiated specimens were divided equally to show very weak & strong differential staining intensity. These differences were found to be statistically significant.

Graph 3: Intergroup comparison of Differential staining intensity with PSR-FG stain

Table 4: Intergroup comparison of Fibre orientation

			Fibre orientation		Total
			Haphazard	Parallel	
Type of SCC	Well differentiated	N	0	20	20
		%	0.0%	100.0%	100.0%
	Moderately differentiated	N	4	16	20
		%	20.0%	80.0%	100.0%
	Poorly differentiated	N	8	12	20
		%	40.0%	60.0%	100.0%
Total		N	12	48	60
		%	20.0%	80.0%	100.0%
P value					0.007, S

Chi square test

Intergroup comparison of Fibre orientation was done using chi square test and a statistically significant difference was found. As the degree of differentiation deteriorated in OSCC, the proportion of haphazard fibre orientation increased.

Graph 4: Intergroup comparison of Fibre orientation

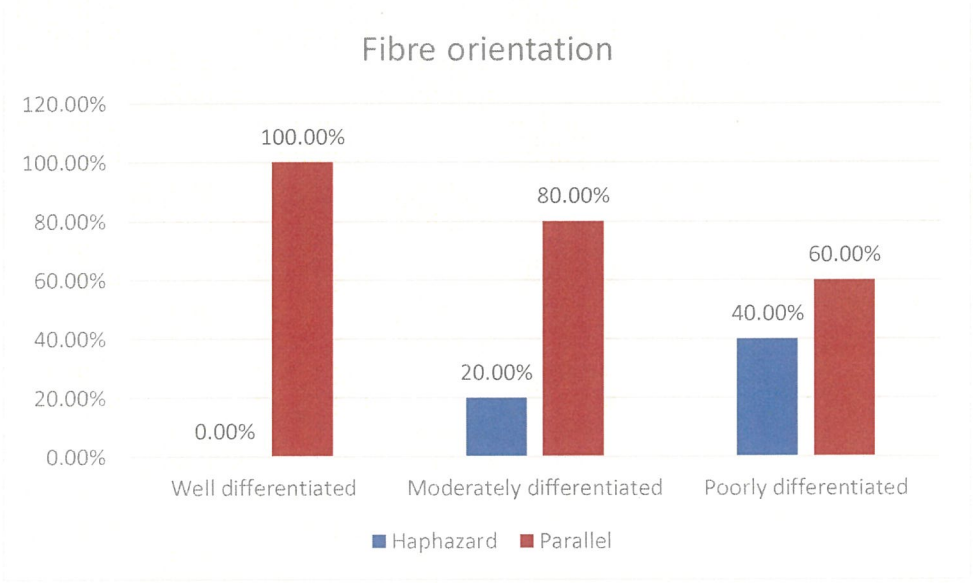

Table 5: Intergroup comparison of Fibre arrangement

			Fibre arrangement		Total
			Dense	Loose	
Type of SCC	Well differentiated	N	8	12	20
		%	40.0%	60.0%	100.0%
	Moderately differentiated	N	4	16	20
		%	20.0%	80.0%	100.0%
	Poorly differentiated	N	4	16	20
		%	20.0%	80.0%	100.0%
Total		N	n	44	60
		%	26.7%	73.3%	100.0%
P value					0.256, NS

Chi square test

Intergroup comparison of Fibre arrangement was done using chi square test and the differences among well, moderately & poorly differentiated OSCC regarding fibre arrangement were not found to be statistically significant. Among all the three types of OSCC, the 'loose' type of arrangement was found to be predominantly present.

Graph 5: Intergroup comparison of Fibre arrangement

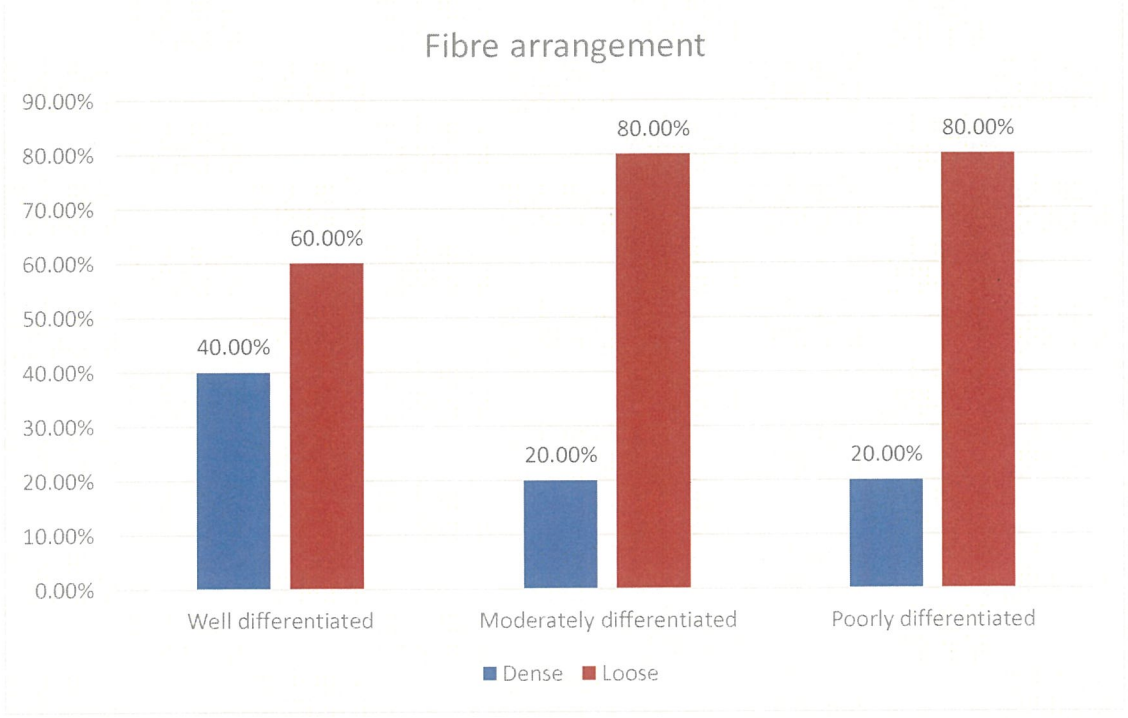

Table 6: Intergroup comparison of Fibre thickness

Fibre thickness					
	N	Mean	Std. Deviation	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound
Well differentiatedSCC	20	5.2880	.83448	4.8975	5.6785
Moderately differentiated SCC	20	3.3360	1.13185	2.8063	3.8657
Poorly differentiated SCC	20	1.7440	.37420	1.5689	1.9191
P value		<0.001, S			
Post hoc pairwise comparison		WDSCC * MDSCC - <0.001, S WDSCC * PDSCC - <0.001, S MDSCC * PDSCC - <0.001, S			

One way ANOVA along with Post hoc Tukey's test

Intergroup comparison of Fibre thickness was done using One way ANOVA test along with post hoc pairwise comparisons by using post hoc Tukey's test. The mean fibre thickness among well differentiated OSCC specimens was found to be significantly more as compared to that among moderately differentiated type, which was further significantly more than that among poorly differentiated type.

Graph 6: Intergroup comparison of Fibre thickness

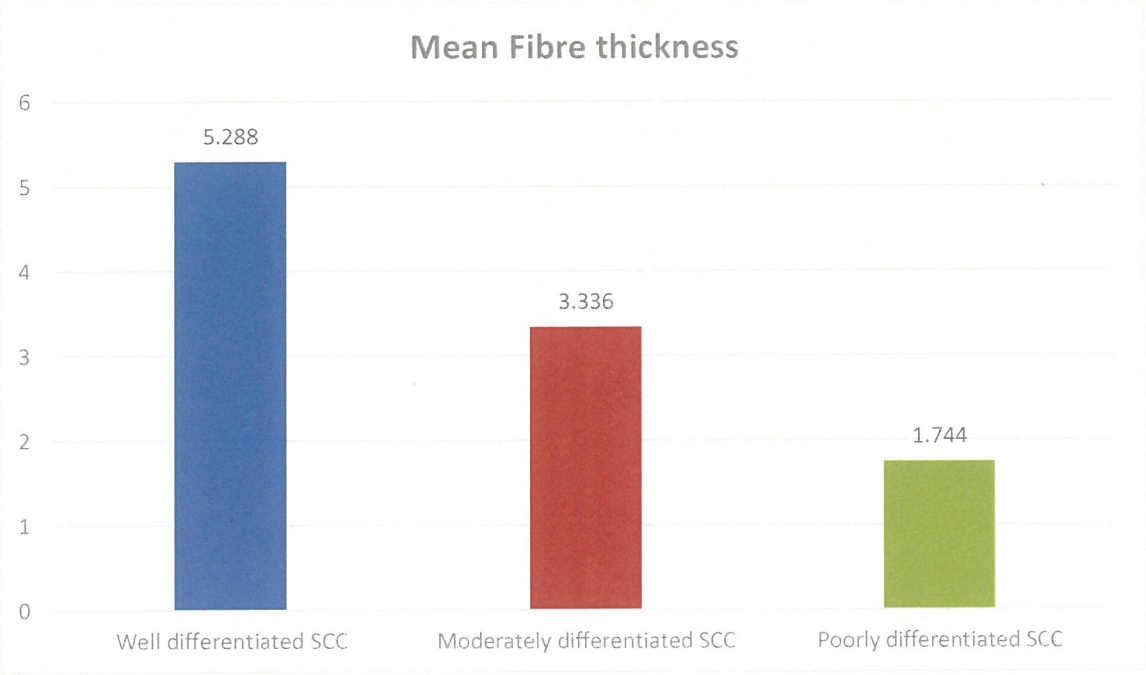

FIG 3: PHOTOMICROGRAPH SHOWING RED-ORANGE HUE OF THICK COLLAGEN FIBERS STAINED WITH PSR IN WELL DIFFERENTIATED SQUAMOUS CELL CARCINOMA, 40X. (POLARISING MICROSCOPY)

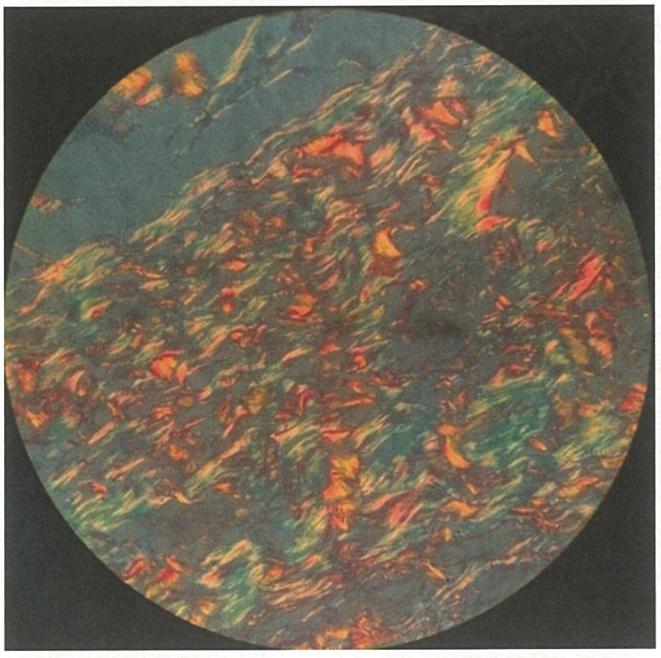

FIG 4: PHOTOMICROGRAPH SHOWING ORANGE-YELLOW HUE OF THICK FIBERS STAINED WITH PSR, IN MODERATELY DIFFERENTIATED SQUAMOUS CELL CARCINOMA, 40X (POLARISING MICROSCOPY)

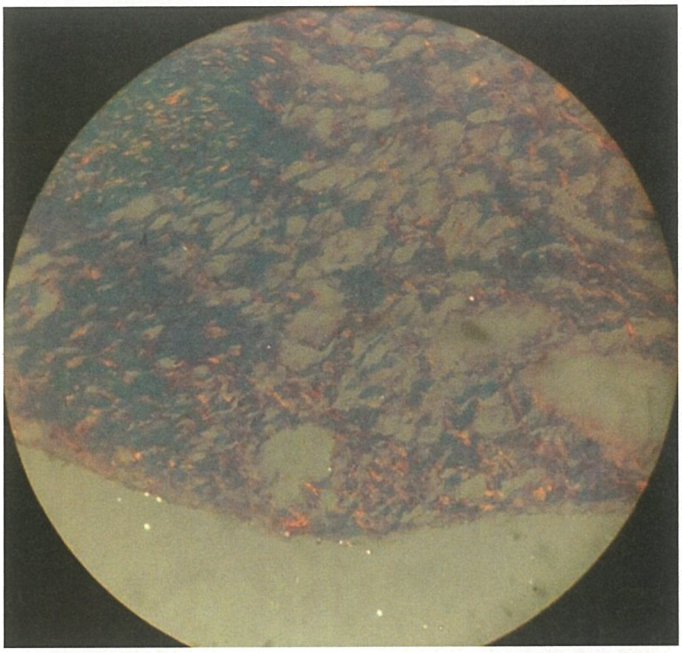

FIG 5: PHOTOMICROGRAPH SHOWING GREEN HUE OF THIN COLLAGEN FIBERS STAINED WITH PSR IN POORLY DIFFERENTIATED SQUAMOUS CELL CARCINOMA,20X (POLARISING MICROSCOPY)

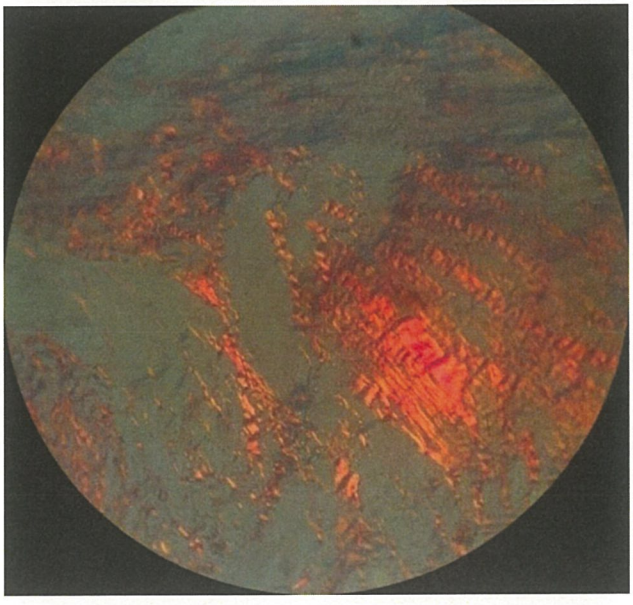

FIG 6: PHOTOMICROGRAPH SHOWING DENSE, PARALLEL ARRANGEMENT OF COLLAGEN FIBERS WITH RED ORANGE BIREFRINGENCE WITH PSR IN WELL DIFFERENTIATED SQUAMOUS CELL CARCINOMA, 40X. (POLARISING MICROSCOPY)

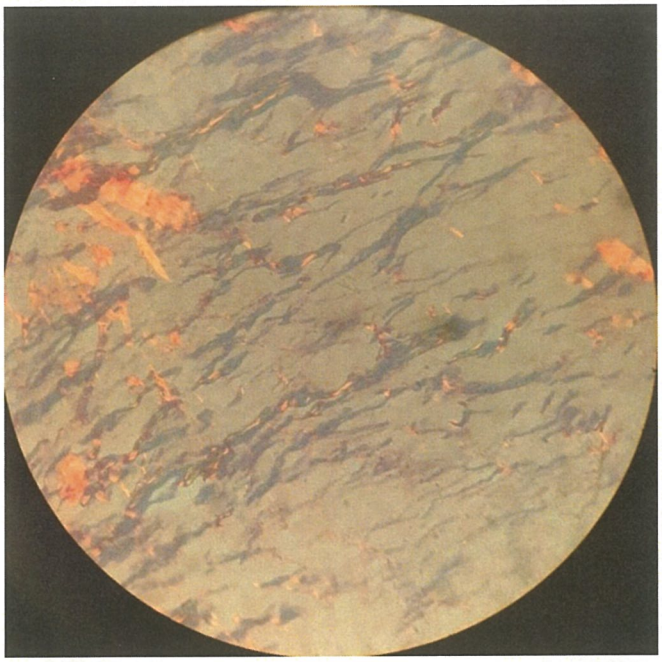

FIG 7: PHOTOMICROGRAPH SHOWING LOOSE, HAPHAZARDLY ARRANGED COLLAGEN FIBERS WITH GREEN BIREFRINGENCE WITH PSR IN POORLY DIFFERENTIATED SQUAMOUS CELL CARCINOMA, 40X. (POLARISING MICROSCOPY)

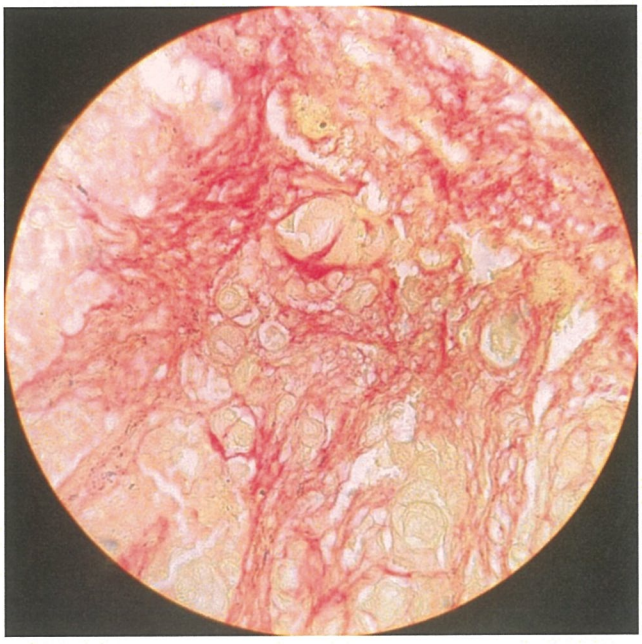

FIG 8: PHOTOMICROGRAPH SHOWING WEAK DIFFERENTIATION OF PSR STAINED COLLAGEN FIBERS AND FAST GREEN STAINED NON – COLLAGENOUS PROTEINS; 20X. (BRIGHTFIELD MICROSCOPY)

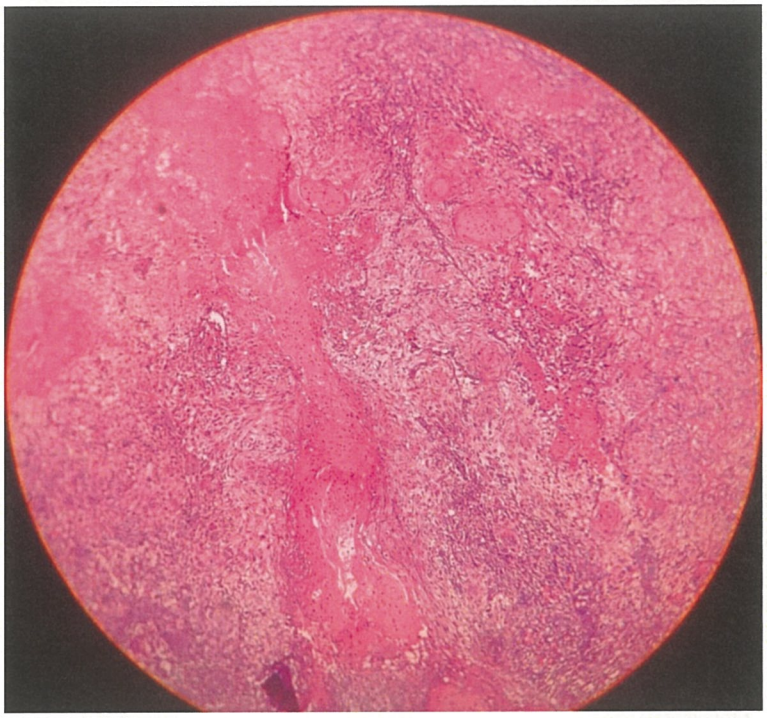

FIG 9: PHOTOMICROGRAPH SHOWING H & E STAINED SECTION OF WELL DIFFERENTIATED SQUAMOUS CELL CARCINOMA.

DISCUSSION

DISCUSSION

Oral cancer is a health concern with a global incidence of 3,77, 713 new cases and 1,77, 757 deaths in 2020[61]. Cancers of the oral cavity ranks sixth in the world. In India, around 219,722 new cases and 1,21,096 deaths were reported in 2020[62].About 60-80% of the cases of oral cancer visit their specialist oncologists at advanced stages and early detection strategies could help to achieve 20% reduction in advanced stage disease[63]. Five-year survival rates in India is around 20% only[64]& this is due to poor understanding of the pathogenesis, failure to detect early and lack of prevention strategies.

Oral Squamous Cell Carcinoma (OSCC) accounts to 84-97% among all oral cancers & pose a heavy burden to healthcare systems[6].

Oral Squamous Cell Carcinoma is a neoplasm of epithelial origin that invades into the stroma. The role of extracellular matrix in the tumor microenvironment of this epithelial malignancy has been widely researched upon, the composition of which determines the prognosis to a large extent. Collagen fibers are the most prominent part of the Extracellular Matrix; therefore have been of prime importance.

Van Geison and Masson's trichrome stains help to differentiate collagen from other tissue components. Amongst the special stains to identify collagen, Picro Sirius Red can reveal very thin collagen fibers. Numerous studies have been published in the past where Picro Sirius Red under polarised light was used to study collagen in Oral Squamous Cell Carcinoma[28,60,39]. In the present study, we chose to use Picro Sirius Red under polarised light as well as combine Picro Sirius Red with Fast Green for viewing under bright field microscopy. Fast green stains non collagenous proteins green, while Picro Sirius Red

stains collagenous proteins red-orange. Only one research has been published in English literature where Picro Sirius Red Fast Green were combined and used to study collagen changes in inflamed rat colon [54]. Therefore, this study is a pioneer with the aim to compare Picro Sirius Red under polarised light in Oral Squamous Cell Carcinoma with PSR-Fast Green combination stain under bright-field microscopy.

We included 10 samples of normal oral mucosa from healthy individuals as controls. These samples were retrieved from archives of routine frenectomy/ operculectomy procedures which were diagnosed as normal oral mucosa. 20 samples each of previously diagnosed Oral Squamous Cell Carcinoma of all grades were retrieved from the archives and 4 micrometer sections were cut. Each section was stained with Picro Sirius Red, PSR-Fast Green and Haemotoxylin/Eosin and visualised under polarised light microscopy and bright field microscopy respectively.

On comparing the hue of Picrosirius red stained collagen fibres under polarised light microscopy, all tissues of the well differentiated group showed an orange red birefringence which was similar to the results in previous studies[28,39,60]. 80% of Moderately differentiated Oral Squamous Cell Carcinoma revealed a yellow orange hue; while 100% of poorly differentiated tissues exhibited a green yellow colour. These differences were statistically significant. (Table 1, Graph1)

Collagen network in normal stroma are usually arranged in bundles. The thick fibers are Type 1 collagen and usually reveal yellowish-orange to orange-red polarization. Thin collagen fibers (Type 3) show green to greenish yellow polarization[65]. As malignant epithelial tumour islands invade the stroma, the collagen fiber bundles seem to become

more thinner and haphazardly arranged. This may be because, poorly differentiated OSCC invades the ECM as isolated cells; secretes collagenolytic enzymes and is more aggressive that it breaks down the thick bundles into thinner fibers. Green- Yellow color may be seen both in thin and thick fibers, suggesting that the collagen is poorly packed whereas the orange red color originates from tightly packed fibers[42]. Change in polarization colors of collagen, may indicate neoplastic progression.

In a study on collagen in odontogenic cysts, Singh HP et al suggested that the greenish-yellow birefringence imparted in Odontogenic Keratocyst can be attributed to the young and immature collagen fibers. Another explanation to PDSCC showing GY hue may be due to the fact that host response to its aggressive nature results in formation of immature collagen[43].

During maturation of collagen fibers, there is change in proteoglycan content of fibers causing dehydration resulting in increase in diameter of collagen fibers and intensity of birefringence. Hence, the change in polarizing colors[66].

Examination of collagen fibers by picrosirius red in conjunction with polarizing microscope can serve as a procedure to differentiate procollagens, intermediate and pathological collagen fibers, which are not tightly packed, from normal packed fibers [67].

To correlate the hue with thickness of fibers, we quantified the fiber thickness using Image J. PSR staining with morphometric image analysis helped us assess collagen qualitatively&quantitatively. Thick fibers were considered to be the ones measuring 2μm -10μm and thin fibers were considered 0.5μm-1.5μm[60].

We found that thick fibers dominated the WDSCC group and thin fibers were more in the PDSCC group. (Table 6, Graph 6). Therefore, correlating the hue and thickness of collagen, we found that thick collagen fibers exhibited an orange red hue and were seen predominantly in WDSCC and with neoplastic progression, PDSCC revealed thin collagen fibrils with green hue. This was in accordance with Venigella and Charu[28]. Ultra microscopic studies have earlier revealed that thick fibers are type 1 collagen while the thin fibers were type 3 collagen[39].

Birefringence is an optical property exhibited by anisotropic materials under polarised light. Collagen is a naturally birefringent substance which is due to the quasi crystalline alignment parallel to the arrangement of its fibers[68]. Sulphonic acid groups of the strong anionic dye Sirius red reacts with cationic groups present in the collagen molecule. The elongated dye molecules are attached to the collagen fibres with their long axes parallel to each other & results in an enhanced birefrengency. Collagen bundles appear green, red or yellow[65]. Several studies have researched this property of collagen using PSR in polarised light[39,60]. In the present study, all cases of WDSCC showed strong collagen birefringence among which 12/20 cases showed extremely strong birefringence. None of the MDSCC or PDSCC were strongly birefringent. However, all PDSCC cases showed weak birefringence. This may be due to sparsely packed collagen content in the thin fibrils as compared to the thick fibers. Previous study by Aeman Khalid et al[66]showed similar results. (Table 2, Graph 2) Puett et al has reported that cross links between fibrils determine the intensity of birefringence[68].

PSR stains collagenous protein content in the Red – Orange – Yellow - Green spectrum depending on the grade of OSCC; but requires specialised polarised light microscopy for better visualisation. Therefore we combined PSR with Fast Green, where FG would stain all non collagenous protein components in green. The differential staining of collagen in red and non collagen in green can be viewed using this combination stain, under bright field microscopy. When combined, the difference in colour was previously reported to be amplified[54]. However in the present study, differentiation between the two components was weak in all grades of Oral Squamous Cell Carcinoma. All grades of OSCC showed uniformly weak staining intensity with PSR-FG. (Table3, Graph 3).

WDSCC as previously discussed showed presence of thick collagen fibers. These fibers were oriented parallel showing fiber maturation and regularity in its deposition. The fiber orientation was more haphazard among PDSCC which showed thinner fibers. Perhaps thickness of the fibers play a role in their orientation. Thick fibers were placed at regular intervals whereas thinner fibers were arranged in a haphazard manner. Thin haphazard fiber arrangement perhaps enhanced the capability of tumour cells to invade more aggressively in PDSCC. In the present study, all WDSCC and 80% MDSCC showed parallel orientation while PDSCC cases showed a mixture of parallel and haphazard arrangement. (Table 4, Graph 4).

Also, we assessed the collagen fibre arrangement and found 73% of all cases showed a loose pattern. Dense pattern was seen in a few WDSCC cases. In the present study, we found a predominantly parallel oriented but loosely arranged collagen network. The possible explanation to the same may be that, invading tumour cells led to secretion of lytic enzymes which perhaps caused break down of intervening non collagenous matrix,

which resulted in loosely arranged appearance of collagen in the matrix. (Table 5, Graph 5).

Collagen influences tumor progression in two different ways. Tumour cells are considered like a foreign body and the host response increases collagen production to cordon off the tumour and prevent it from spread. In a different scenario, collagen degradation and decreased synthesis allow invasion of tumor cells through the stroma. Degradation of the extracellular matrix is dependent on specific interactions between tumor and host cells[25].

The particular colours produced by polarization microscopy of PSR stained section could be due to fibre size, alignment and packing, cross linking of fibres, interstitial ground substance and water content. It is also seen that in tightly packed and better aligned collagen molecules, showed a shift to the longer wavelength of polarization colours [66,69,70].

In the present study, we found thick parrallelly arranged strongly birefringent dense collagen fibres showing predominantly orange red polarisation in well differentiated OSCC's while poorly differentiated OSCC's revealed thin loosely arranged weakly birefriengent haphazard collagen fibres.

We believe that collagen acts as a barrier which prevents spread of tumour cells in the surrounding stroma. Collagen degradation results from secretion of collagenolytic proteins by the tumour cells; hence these findings can be used to predict the tumour behavior and its possible response to treatment. Also it gives an insight to probably develop treatment strategies which can be based upon stromal modulation.

CONCLUSION

CONCLUSION

The present study was conducted using 20 samples each of previously diagnosed Oral Squamous Cell Carcinoma of all grades which were retrieved from the archives. 4 micrometer sections were cut & each section was stained with Picrosirius Red, Picrosirius Red-Fast Green and Hematoxylin/Eosin and visualised under polarised light microscopy and bright field microscopy respectively. We included 10 samples of normal oral mucosa from healthy individuals as controls. These samples were retrieved from archives of routine frenectomy/ operculectomy procedures which were diagnosed as normal oral mucosa.

We compared collagen fibers for their qualitative and quantitative characteristics in various grades of Oral Squamous Cell Carcinoma using Picrosirius Red, Picrosirius Red-Fast Green stains under Polarised and bright field microscopy respectively along with Image analysis.

We observed thick, parallelly arranged, strongly birefringent dense collagen fibres showing predominantly orange red polarization in Well Differentiated Oral Squamous Cell Carcinoma while poorly differentiated Oral Squamous Cell Carcinoma revealed thin loosely arranged weakly birefringent haphazard collagen fibers.

It can be concluded that collagen acts as a barrier and prevents spread of tumour cells in the stroma. This property can be used to modulate the stromal compostion which in turn can modify the epithelial tumour behaviour.

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BIBLIOGRAPHY

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APPENDICES

APPENDICES

ANNEXURE – I

BABU BANARASI DAS UNIVERSITY

BBD COLLEGE OF DENTAL SCIENCES, LUCKNOW

INSTITUTIONAL RESEARCH COMMITTEE APPROVAL

The project titled **"Assessment Of Collagen Birefringence In Different Grades Of OSCC Using Picrosirius Red-Polarizing Microscopy And Comparison With PSR-FAST Green Stain"** submitted by **Dr Deba Kumar Das** Postgraduate student in the **Department of Oral Pathology & Microbiology** for the Thesis Dissertation as part of MDS Curriculum for the academic year 2021-2024 with the accompanying proforma was reviewed by the Institutional Research Committee in its meeting held on **14th September, 2022** at BBDCODS.

The Committee has granted approval on the scientific content of the project. The proposal may now be reviewed by the Institutional Ethics Committee for granting ethical approval.

Prof. Dr. Puneet Ahuja
Chairperson

Dr. Mona Sharma
Co-Chairperson

ANNEXURE – II

BABU BANARASI DAS UNIVERSITY
BBD COLLEGE OF DENTAL SCIENCES, LUCKNOW

BBDCODS/IEC/09/2022 **Dated: 16th September, 2022**

Communication of the Decision of the Xth Institutional Ethics Sub-Committee Meeting

IEC Code: 37

Title of the Project: Assessment Of Collagen Birefringence In Different Grades Of OSCC Using Picrosirius Red-Polarizing Microscopy And Comparison With PSR-FAST Green Stain.

Principal Investigator: Dr Deba Kumar Das **Department:** Oral Pathology & Microbiology

Name and Address of the Institution: BBD College of Dental Sciences Lucknow.

Type of Submission: New, MDS Project Protocol

Dear Dr Deba Kumar Das,

The Institutional Ethics Sub-Committee meeting comprising following members was held on 15th September, 2022.

1.	Dr. Lakshmi Bala Member Secretary	Prof. and Head, Department of Biochemistry
2.	Dr. Praveen Singh Samant Member	Prof. & Head, Department of Conservative Dentistry & Endodontics
3.	Dr. Jiji George Member	Prof. & Head, Department of Oral Pathology & Microbilogy
4.	Dr. Amrit Tandan Member	Professor, Department of Prosthodontics and Crown & Bridge
5.	Dr. Rana Pratap Maurya Member	Reader, Department of Orthodontics & Dentofacial Orthopaedics

The committee reviewed and discussed your submitted documents of the current MDS Project Protocol in the meeting.
The comments were communicated to PI, thereafter it was revised.

Decisions: The committee approved the above protocol from ethics point of view.

Forwarded by:

Prof. Dr. Puneet Ahuja
Principal
BBD College of Dental Sciences
BBD University, Lucknow

PRINCIPAL
Babu Banarasi Das College of Dental Sciences
(Babu Banarasi Das University)
BBD City, Faizabad Road, Lucknow-226028

Lakshmi Bala 16/09/22

Dr. Lakshmi Bala
Member-Secretary
Institutional Ethics Sub-Committee (IEC)
BBD College of Dental Sciences
BBD University, Lucknow

Member-Secretary
Institutional Ethic Committee
BBD College of Dental Sciences
BBD University,
Faizabad Road, Lucknow-226028

ANNEXURE – III

Observations

HUE PSR				
	CASES	Orange Red	Yellow Orange	Green Yellow
Well Differentiated SCC	1	y	-	-
	2	Y	-	-
	3	Y	-	-
	4	Y	-	-
	5	Y	-	-
	6	Y	-	-
	7	Y	-	-
	8	Y	-	-
	9	Y	-	
	10	Y	-	-
	11	Y	-	-
	12	Y	-	-
	13	Y	-	-
	14	Y	-	-
	15	Y	-	-
	16	Y	-	-
	17	Y	-	-
	18	Y	-	-
	19	Y	-	-
	20	y	-	-
	CASES	Orange Red	Yellow Orange	Green Yellow
Moderately Differentiated SCC	1	-	Y	-
	2	-	Y	-
	3	-	Y	-
	4	Y	-	-
	5	-	Y	-
	6	-	Y	-
	7	-	Y	-
	8	-	Y	-
	9	Y	-	-
	10	-	Y	-
	11	-	Y	-
	12	-	Y	-
	13	-	Y	-
	14	Y	-	-
	15	-	Y	-
	16	-	Y	-
	17	-	Y	-
	18	Y	-	-
	19	-	Y	-
	20	-	Y	-

	CASES	Orange Red	Yellow Orange	Green Yellow
Poorly Differentiated SCC	1	-	-	Y
	2	-	-	Y
	3	-	-	Y
	4	-	-	Y
	5	-	-	Y
	6	-	-	Y
	7	-	-	Y
	8	-	-	Y
	9	-	-	Y
	10	-	-	Y
	11	-	-	Y
	12	-	-	Y
	13	-	-	Y
	14	-	-	Y
	15	-	-	Y
	16	-	-	Y
	17	-	-	Y
	18	-	-	Y
	19	-	-	Y
	20	-	-	Y

BIREFRINGENCE					
			PSR		PSR - FG
	CASES	Strong	Weak	Strong	Weak
Well Differentiated SCC	1	Y	-	YY	-
	2	Y	-	YY	-
	3	YY	-	-	-
	4	YY	-	-	-
	5	YY	-	-	-
	6	Y	-	YY	-
	7	Y	-	YY	-
	8	YY	-	-	-
	9	Y	-	YY	-
	10	Y	-	YY	-
	11	YY	-	-	-
	12	YY	-	-	-
	13	YY	-	-	-
	14	YY	-	-	-
	15	Y	-	YY	-
	16	Y	-	YY	-
	17	YY	-	-	-
	18	YY	-	-	-
	19	YY	-	-	-
	20	YY	-	-	-
Moderately Differentiated SCC	CASES	Strong	Weak	Strong	Weak
	1	-	Y	Y	-
	2	-	Y	-	Y
	3	-	Y	-	YY
	4	Y	-	-	Y
	5	Y	-	Y	Y
	6	-	Y	-	-
	7	-	Y	-	Y
	8	-	Y	-	YY
	9	-	Y	Y	-
	10	-	Y	-	Y
	11	-	Y	-	YY
	12	Y	-	-	Y
	13	Y	-	-	Y
	14	Y	-	-	Y
	15	-	Y	Y	-
	16	-	Y	-	Y
	17	Y	-	-	Y
	18	Y	-	-	Y
	19	Y	-	-	Y
	20	-	Y	-	YY

	CASES	Strong	Weak	Strong	Weak
Poorly Differentiated SCC	1	Y	-	YY	-
	2	Y	-	YY	-
	3	Y	-	-	Y
	4	Y	-	-	Y
	5	Y	-	-	Y
	6	Y	-	YY	-
	7	Y	-	YY	-
	8	Y	-	-	Y
	9	Y	-	-	Y
	10	Y	-	YY	-
	11	Y	-	-	Y
	12	Y	-	YY	-
	13	Y	-	-	Y
	14	Y	-	-	Y
	15	Y	-	YY	-
	16	Y	-	-	Y
	17	Y	-	-	Y
	18	Y	-	-	Y
	19		Y	-	Y
	20	Y	-	YY	-

	CASES	Thin fibers (0.5 -1.5 mm)	Thick fibers (2 – 10.0 mm width)
Well Differentiated SCC	1	-	6.6 , 6.7 , 2.5 , 2.8 , 4.1
	2	-	5.8 , 7.5 , 5.2 , 6.5 , 5.2
	3	-	6.8 , 4.0 , 2.2 , 6.8 , 5.8
	4	-	6.5 , 5.8 , 4.9 , 9.9 , 5.8
	5	-	4.0 , 4.1 , 4.0 , 2.4 , 4.5
	6	-	6.6 , 6.7 , 2.5 , 2.8 , 4.1
	7	-	5.8 , 7.5 , 5.2 , 6.5 , 5.2
	8	-	6.8 , 4.0 , 2.2 , 6.8 , 5.8
	9	-	6.5 , 5.8 , 4.9 , 9.9 , 5.8
	10	-	4.0 , 4.1 , 4.0 , 2.4 , 4.5
	11	-	6.8 , 4.0 , 2.2 , 6.8 , 5.8
	12	-	6.6 , 6.7 , 2.5 , 2.8 , 4.1
	13	-	6.5 , 5.8 , 4.9 , 9.9 , 5.8
	14	-	5.8 , 7.5 , 5.2 , 6.5 , 5.2
	15	-	4.0 , 4.1 , 4.0 , 2.4 , 4.5
	16	-	6.5 , 5.8 , 4.9 , 9.9 , 5.8
	17	-	4.0 , 4.1 , 4.0 , 2.4 , 4.5
	18	-	6.8 , 4.0 , 2.2 , 6.8 , 5.8

	19	-	6.6 , 6.7 , 2.5 , 2.8 , 4.1
	20	-	5.8 , 7.5 , 5.2 , 6.5 , 5.2

	CASES	Thin fibers (0.5 -1.5 mm)	Thick fibers (2 – 10.0 mm width)
Moderately Differentiated SCC	1	-	**2.8 , 2.5 , 2.5 , 2.5 , 3.3**
	2	1.6 , 1.6	2.4 , 2.4 , 4.0
	3	1.6 , 1.6 , 1.6	2.0 , 4.8
	4	-	4.4 , 5.9 , 5,4 , 3.8 , 6.1
	5	1.7	5.7 , 5.3 , 3.9 , 4.0
	6	-	**2.8 , 2.5 , 2.5 , 2.5 , 3.3**
	7	1.6 , 1.6	2.4 , 2.4 , 4.0
	8	1.6 , 1.6 , 1.6	2.0 , 4.8
	9	-	4.4 , 5.9 , 5,4 , 3.8 , 6.1
	10	1.7	5.7 , 5.3 , 3.9 , 4.0
	11	1.6 , 1.6	2.4 , 2.4 , 4.0
	12	-	**2.8 , 2.5 , 2.5 , 2.5 , 3.3**
	13	-	4.4 , 5.9 , 5,4 , 3.8 , 6.1
	14	1.6 , 1.6 , 1.6	2.0 , 4.8
	15	1.6 , 1.6	2.4 , 2.4 , 4.0
	16	1.7	5.7 , 5.3 , 3.9 , 4.0
	17	-	4.4 , 5.9 , 5,4 , 3.8 , 6.1
	18	-	**2.8 , 2.5 , 2.5 , 2.5 , 3.3**
	19	1.6 , 1.6 , 1.6	2.0 , 4.8
	20	1.7	5.7 , 5.3 , 3.9 , 4.0

	CASES	Thin fibers (0.5 -1.5 mm)	Thick fibers (2 – 10.0 mm width)
Poorly Differentiated SCC	1	0.7 , 0.7 , 1.7 , 0.7	2.2
	2	1.8 , 0.8	3.4 , 2.2 , 2.2
	3	0.7 , 1.0 , 0.7	2.3 , 2.4
	4	1.7 , 1.6 , 1.1	2.5 , 3.5
	5	1.1 , 1.1	2.5 , 2.2 , 2.8
	6	0.7 , 0.7 , 1.7 , 0.7	2.2
	7	1.8 , 0.8	3.4 , 2.2 , 2.2
	8	0.7 , 1.0 , 0.7	2.3 , 2.4
	9	1.7 , 1.6 , 1.1	2.5 , 3.5
	10	1.1 , 1.1	2.5 , 2.2 , 2.8

	11	1.8 , 0.8	3.4 , 2.2 , 2.2
	12	0.7 , 0.7 , 1.7 , 0.7	2.2
	13	1.1 , 1.1	2.5 , 2.2 , 2.8
	14	1.7 , 1.6 , 1.1	2.5 , 3.5
	15	0.7 , 1.0 , 0.7	2.3 , 2.4
	16	1.7 , 1.6,1.1	2.5 , 3.5
	17	1.1 , 1.1	2.5 , 2.2 , 2.8
	18	1.8 , 0.8	3.4 , 2.2 , 2.2
	19	0.7 , 1.0 , 0.7	2.3 , 2.4
	20	0.7 , 0.7 , 1.7 , 0.7	2.2

FIBER ORIENTATION AND ARRANGEMENT					
Arrangement		Fiber Orientation		Fiber	
	CASES	Haphazard	Parallel	Dense	Loose
Well Differentiated SCC	1	-	Y	Y	-
	2	-	Y	Y	-
	3	-	Y	Y	-
	4	-	Y	Y	-
	5	-	Y	Y	-
	6	-	Y	Y	-
	7	-	Y	Y	-
	8	-	Y	Y	-
	9	-	Y	Y	-
	10	-	Y	Y	-
	11	-	Y	Y	-
	12	-	Y	Y	-
	13	-	Y	Y	-
	14	-	Y	Y	-
	15	-	Y	Y	-
	16	-	Y	Y	-
	17	-	Y	Y	-
	18	-	Y	Y	-
	19	-	Y	Y	-
	20	-	Y	Y	-

	CASES	Haphazard	Parallel	Dense	Loose
Moderately Differentiated	1	-	Y	-	Y
	2	Y	-	-	Y
	3	-	Y	-	Y
	4	-	Y	Y	-
	5	-	Y	-	Y
	6	-	Y	-	Y
	7	Y	-	-	Y

SCC	8	-	Y	-	Y
	9	-	Y	Y	-
	10	Y	-	-	Y
	11	-	Y	-	Y
	12	-	Y	-	Y
	13	-	Y	-	Y
	14	-	Y	-	Y
	15	-	Y	Y	-
	16	-	Y		Y
	17	-	Y	Y	-
	18	Y	-	-	Y
	19	-	Y	-	Y
	20	-	Y	-	Y

	CASES	Haphazard	Parallel	Dense	Loose
Poorly Differentiated SCC	1	Y	-	-	Y
	2	Y	-	-	Y
	3	-	Y	-	Y
	4	-	Y	Y	-
	5	-	Y	-	Y
	6	Y	-	-	Y
	7	Y	-	-	Y
	8	-	Y	-	Y
	9	-	Y	Y	-
	10	Y	-	-	Y
	11	-	Y	-	Y
	12	Y	-	-	Y
	13	-	Y	Y	-
	14	-	Y	-	Y
	15	-	Y	-	Y
	16	Y	-	-	Y
	17	Y	-	-	Y
	18	-	Y	Y	-
	19	-	Y	-	Y
	20	-	Y	-	Y

LIGHT MICROSCOPY		HUE	
Well Differentiated SCC	CASES	PSR	PSR – FG
	1	Red Orange	Light Green
	2	Red Orange	Light Green
	3	Red Orange	Light Green
	4	Red Orange	Light Green
	5	Red Orange	Light Green
	6	Red Orange	Light Green
	7	Red Orange	Light Green

	8	Red Orange	Light Green
	9	Red Orange	Light Green
	10	Red Orange	Light Green
	11	Red Orange	Light Green
	12	Red Orange	Light Green
	13	Red Orange	Light Green
	14	Red Orange	Light Green
	15	Red Orange	Light Green
	16	Red Orange	Light Green
	17	Red Orange	Light Green
	18	Red Orange	Light Green
	19	Red Orange	Light Green
	20	Red Orange	Light Green

	CASES	PSR	PSR-FG
Moderately Differentiated SCC	1	Orange Yellow	Light Green
	2	Orange Yellow	Light Green
	3	Orange Yellow	Light Green
	4	Orange Yellow	Light Green
	5	Orange Yellow	Light Green
	6	Orange Yellow	Light Green
	7	Orange Yellow	Light Green
	8	Orange Yellow	Light Green
	9	Orange Yellow	Light Green
	10	Orange Yellow	Light Green
	11	Orange Yellow	Light Green
	12	Orange Yellow	Light Green
	13	Orange Yellow	Light Green
	14	Orange Yellow	Light Green
	15	Orange Yellow	Light Green
	16	Orange Yellow	Light Green
	17	Orange Yellow	Light Green
	18	Orange Yellow	Light Green
	19	Orange Yellow	Light Green
	20	Orange Yellow	Light Green

	CASES	PSR	PSR – FG
Poorly Differentiated SCC	1	Green	Light Green
	2	Green	Light Green
	3	Green	Light Green
	4	Green	Light Green
	5	Green	Light Green
	6	Green	Light Green
	7	Green	Light Green
	8	Green	Light Green
	9	Green	Light Green
	10	Green	Light Green

	11	Green	Light Green
	12	Green	Light Green
	13	Green	Light Green
	14	Green	Light Green
	15	Green	Light Green
	16	Green	Light Green
	17	Green	Light Green
	18	Green	Light Green
	19	Green	Light Green
	20	Green	Light Green

ANNEXURE – IV

Formula Used For The Analysis

Data was entered into Microsoft Excel spreadsheet and then checked for any missing entries. It was analysed using Statistical Package for Social Sciences (SPSS) version 21. Categorical variables were summarized as frequencies and continuous variables were summarized as mean and standard deviation.Graphs were prepared on Microsoft Excel.

Inferential statistics were performed using **Chi-square test & One way Analysis of Variance along with post hoc Tukey's test.Chi-square** test is used to compare categorical data. **One way analysis of variance** test was used to compare more than 2 independent means. Post hoc pairwise comparison was done using **Post hocTukey's test**. The level of statistical significance was set at 0.05.

The following formulas were employed for calculation for various parameters:

Mean

The mean (also known as average), is obtained by dividing the sum of observed values by the number of observations, *n*. The formula for the mean is given below as :

$$\bar{X} = \frac{\sum_{i=1}^{i=n} X_i}{n}$$

Where, $\bar{X}$ = Mean

$\sum X$ = Sum of all individual observations

n = Total number of observations

Standard Deviation

The standard deviation gives an idea of how close the entire set of data is to the average value. Data sets with a small standard deviation have tightly grouped, precise data. Data sets with large standard deviations have data spread out over a wide range of values. The formula for standard deviation is given below as:

$$\sigma = \sqrt{\frac{1}{n-1}\sum_{i=1}^{i=n}(X_i - \bar{X})^2}$$

Where, σ = Standard deviation

$\overline{X}$ = Mean

Xi = Individual observation

∑ X = Sum of all individual observations

∑(Xi $\overline{-}$ X) = Sum of differences of every observation from the mean value

n = Total number of observations

Chi square test:

$$\chi^2 = \sum \frac{(observed - expected)^2}{expected}$$

The chi-square statistic measures the difference between actual and expected counts in a statistical experiment. These experiments can vary from two way tables to multinomial experiments. The actual counts are from observations, the expected counts are typically determined from probabilistic or other mathematical models.

Analysis of Variance Test:

This test is used to compare more than two means simultaneously. The critical ratio which is calculated for performing ANOVA test is called as F ratio. It is calculated as ratio of between-groups variance to within groups variance.

$$\text{F ratio} = \frac{\text{Between-groups variance}}{\text{Within-groups variance}}$$

$$\text{F ratio} = \frac{\text{Mean square Between-groups}}{\text{Mean square Within-groups}}$$

Post hoc Tukey's test:

Tukey's post-hoc test is a method that is used to determine which groups among the sample have significant differences. This method calculates the difference between the means of all the groups. Tukey's test values are number which acts as a distance between the groups.

Formula is as follows:

$$\frac{M_1 - M_2}{\sqrt{MS_w\left(\frac{1}{n}\right)}}$$

M = treatment/group mean

n = number per treatment/group

ANNEXURE – V

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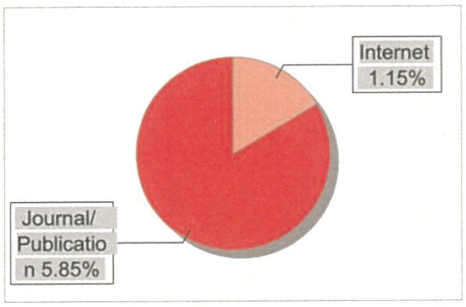

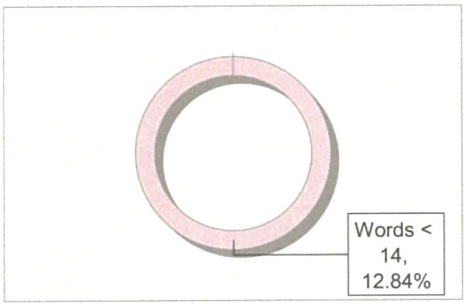

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