"EXPRESSION OF ENDOGLIN IN DIFFERENT GRADES OF ORAL SQUAMOUS CELL CARCINOMA AND ITS CORRELATION WITH MAST CELLS: A RETROSPECTIVE STUDY"

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In

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By

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

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LIST OF ABBREVIATIONS

OSCC	Oral Squamous Cell Carcinoma
WDSCC	Well Differentiated Squamous Cell Carcinoma
MDSCC	Moderately Differentiated Squamous Cell Carcinoma
PDSCC	Poorly Differentiated Squamous cell Carcinoma
WHO	World Health Organization
CD34	Cluster of Differentiation 34
CD30	Cluster of Differentiation 30
CD31	Cluster of Differentiation 31
OED	Oral Epithelial Dysplasia
OPMD	Oral Potentially Malignant Disorder
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelia Growth Factor Receptor
CD105	Cluster of Differentiation 105 (Endoglin)
MVD	Mean Vascular Density / Microvessel Density
MCD	Mast Cell Density
MC	Mast Cell
TGF-β	Transforming Growth Factor Beta
ECM	Extracellular Matrix
PG	Pyogenic Granuloma
HRP IHC	Horseradish Peroxidase Immunohistochemistry
DAB	Diaminobenzidine
RGD	Arg-Gly-Asp (Tripeptide)
hTERT	Human Telomerase Reverse Transcriptase
CK	Cytokeratin
EGFR	Epidermal Growth Factor Receptor
bFGF	Basic Fibroblast Growth Factor
MMP-9	Matrix Metalloproteinase 9
COX	Cyclooxygenase
PAI-1	Plasminogen Activator Inhibitor-1
uPA	Urokinase-Type Plasminogen Activator
TSP-1	Thrombospondin-1

ALK1	Activin receptor-like kinase 1
TbetaR-II	TGF-β Receptor II
Smad	Smad Proteins, Involved in TGF-β Signaling
HHT1	Hereditary Hemorrhagic Telangiectasia Type 1
EGM	Extracellular Matrix
TNF-α	Tumor Necrosis Factor Alpha
IL-8	Interleukin-8
DPX	Dibutylphthalate Polystyrene Xylene

ABSTRACT

ABSTRACT

Background and Objectives:

Oral squamous cell carcinoma (OSCC), arising from the oral mucosa constitutes more than 90% of oral malignancies. Though OSCC is an epithelial malignancy, the invading tumour components are believed to be influenced by the vascularity of surrounding stroma. Among the multiple steps in tumor progression and metastasis, angiogenesis plays a prime role. Angiogenesis can be determined histologically by the immunohistochemical expression of endothelial marker like Endoglin (CD105), a potent pleiotropic angiogenic factor. Many studies suggest that mast cells are immune cell which play an active role in angiogenesis. Quantitative analysis of angiogenesis can be determined by mean vascular density (MVD) and the number of mast cells. Hence, this retrospective and comparative study was conducted to assess the microvascular density using expression of Endoglin and mast cells density using Toluidine Blue in the different grades of tumor stroma.

Materials and Methods:

10 cases each of well differentiated, moderately differentiated, poorly differentiated squamous cell carcinoma and pyogenic granuloma as control, were immunohistochemically analysed for microvascular density using expression of Endoglin (CD 105) and mast cell density using toluidine blue stain.

Results: We observed increased microvascular density which corresponded to the increase in angiogenesis with higher grades of Oral Squamous Cell Carcinoma. Also, we found a decrease in Mast cell density with increasing grades of Oral Squamous cell Carcinoma.

Conclusion: Angiogenesis plays an important role in the progression of Oral Squamous Cell Carcinoma while the role of mast cells remains controversial.



INTRODUCTION



INTRODUCTION

Globally, oral cancer is the sixth most common type of cancer in India, contributing to almost one- third of the total cancer 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. Despite the advances of therapeutic approaches, morbidity and mortality of OSCC have not improved significantly in 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.

Though OSCC is an epithelial malignancy, the invading tumour components are believed to be influenced by vascularity of surrounding stroma. 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.

Neo-angiogenesis makes available all the nutrients required for the tumour growth. Angiogenesis can be quantified by the microvessel density in a high power field and can be considered proportional to the tumor growth. Endoglin is a cluster differentiation factor which is associated with tumour vasculature and its expression can be measured. Similarly, mast cells are immune cells which play an active role in angiogenesis and can be quantified using easily available metachromatic stains like toludine blue.

Therefore, we designed this retrospective and comparative study to assess the expression of Endoglin in different grades of oral SCC with pyogenic granuloma as controls, and to compare the expression of mast cells using toludine blue stain. The

results of this study would probably contribute to better understand the role of angiogenesis in the biological behavior of OSCC's and help develop tumor angiogenesis as a novel target for anticancer therapy.

AIM AND OBJECTIVES

AIM & OBJECTIVES

Aim:

To determine the Mean Vascular Density using Endoglin (CD105) and assess the mast cell density using Toluidine Blue in different grades of OSCC's and evaluating their relationship with the biologic behaviours of these lesions.

Objectives:

1. To determine and compare the Mean Vascular Density in various grades of OSCC's using CD105 (Endoglin).
2. To determine and compare the Mast Cell Density in various grades of OSCC's using Toluidine Blue Stain.
3. To compare the Mean Vascular Density using Endoglin and Mast cell density using Toluidine blue in different grades of OSCC.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

ORAL SQUAMOUS CELL CARCINOMA

Oral squamous cell carcinoma (OSCC), the 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 occurred worldwide in 2012. Amongst which 300, 400 new cancer cases were reported in lip and oral cavity, with 145, 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].

GRADING OF ORAL SQUAMOUS CELL CARCINOMA

Broder's (1920) classification[8]:

According to this classification, tumors were graded on the basis of degree of differentiation and keratinization of tumor cells into:

Grade I: Well differentiated tumors – 75-100% of cells are differentiated

Grade II: Moderately differentiated tumors – 50- 75% of cells are differentiated

Grade III: Poorly differentiated tumors – 25-50% of cells are differentiated

Grade IV: Anaplastic tumor – 0-25% of cells are differentiated

Anneroth's et al (1987) multifactorial grading system[8]:

According to this system, three parameters reflecting tumor cell features including keratinization, nuclear pleomorphism, and mitoses were evaluated in the whole thickness of the tumor and each scored from 1-4 (Table 1).

Pattern of invasion, stage of invasion, and lymphoplasmacytic infiltration representing tumor-host relationship were graded in the most invasive margins and scored from 1-4. Then the sum of scores were grouped as follows: 6-12 grade I, 13-18 grade II, 19-24 grade III, and the results were compared in the metastasizing and non-metastasizing groups.

Anneroth's et al (1987) multifactorial grading system for oral SCCs (Table1)[8]:

Morphologic Parameter	1 Point	2 Points	3 Points	4 Points
Degree of keratinization	>50% cells Keratinized	20–50% cells Keratinized	5–20% cells keratinized	0–5% cells keratinized
Nuclear pleomorphism	Little nuclear pleomorphism	Moderately abundant nuclear pleomorphism	Abundant nuclear pleomorphism	Extreme nuclear pleomorphism
Number of mitosis / hpf	0–1	2–3	4–5	>5
Pattern of invasion	Pushing, well-delineated infiltrating borders	Infiltrating, solid cords, bands and/or strands	Small groups or cords of infiltrating cells	Marked and widespread cellular dissemination in small groups and/or in single Cells
Stage of invasion	Carcinoma-in-situ and/or questionable invasion	Distinct invasion, but involving lamina propria only	Invasion below lamina propria adjacent to muscles, salivary gland tissues, and periosteum	Extensive and deep invasion replacing most of the stromal tissue and infiltrating jaw Bone
Lymphoplasmacytic infiltration	Marked	Moderate	Slight	None

Bryne's et al (1992)[8]

Deep invasive cell grading system:

According to this system, number of mitosis and stage of invasion was omitted from the Anneroth's grading system, while the rest of the 4 parameters mentioned above were measured in the deepest invasive margins, and not in the whole thickness of the tumor, and graded similarly. The sum of scores were grouped as follows: 4-8 grade I, 9-12 grade II, 13-16 grade III, and the results were compared in the metastasizing and non-metastasizing groups. In cases where opinion of the two authors differed, the disagreement was resolved by consensus after joint review using a multiheaded microscope, and reviewed by the third author. The results of the three grading systems in each of the two groups (metastatic and non-metastatic) were analyzed by logistic regression[8].

The incidence rate of oral cancer varies widely throughout the world because of adapting cancer associated behaviors. OSCC has a multifactorial etiology like use of tobacco, alcohol, betel quid chewing, HPV infection, actinic radiation, poor oral hygiene, iron deficiency, syphilis and candidiasis etc. Amongst these, tobacco habit and excessive alcohol consumption accounted for about 90% of oral cancers[9].

OSCC is recognised as a disease ensuing from the genetic damage, leading to unrestrained cell proliferation of the damaged cells[10].

There are multiple steps that are associated with tumour progression, of them one is neo- angiogenesis, which is one of the essential factor for growth of tumour and its metastasis[11].

An important predictor of tumour behavior is the intra tumour micro vessel density (MVD). The intensity of angiogenesis has been associated with poor prognosis in

several cancers cases. Angiogenesis is determined by immunohistochemical assessment and with several endothelial cell markers, in which Endoglin CD105 is a potent pleiotropic angiogenic factor expressed on activated endothelial cells during angiogenesis, is considered to be a specific marker for the detection of tumour angiogenesis[12].

ANGIOGENESIS AND CARCINOMA DEVELOPMENT

Angiogenesis, defined as the growth of new blood vessels (neovascularization) from pre-existing ones[13]. It is a complex process, needed for the continued growth and survival of solid neoplasia. The process of angiogenesis is in itself is a multi-step process involving extensive interplay between cells, soluble factors, and extracellular matrix (ECM) components. Sequential steps in angiogenesis include: degradation of basement membrane by proteases; migration of endothelial cells into the interstitial space and sprouting; endothelial cell proliferation at the migrating tip; lumen formation, generation of new basement membrane with the recruitment of pericyte, formation of anastomoses and finally the blood flow[14].

Under normal circumstances, angiogenesis is a highly ordered process under tight regulation, and it requires inducing quiescent endothelial cells in a monolayer to divide and spread the vascular network only to the extent demanded by demands of growing tissues. Many positively and negatively acting factors that influence angiogenesis are- soluble polypeptides, cell-cell and cell- matrix interactions, and hemodynamic effects. Some of the soluble growth factors, membrane- bound molecules, and mechanical forces that mediate these signals are VEGF, TGF- beta, EGF, Ang1, Ang 2, TNF-alpha, Angiogenin, Angiotropin etc. Amongest these, most well-characterized angiogenic factor is vascular endothelial growth factor (VEGF),

which, increases endothelial cell permeability, stimulates endothelial cell uPA/PAI-1 production, stimulates endothelial cell proliferation, inhibits endothelial cell apoptosis, enhances endothelial cell migration and stimulates in vivo angiogenesis[15].

Angiogenesis can also be determined histologically by the immunohistochemical expression of various endothelial markers like CD31, CD34 and Endoglin etc. In a process very similar to normal angiogenesis, a tumor may elicit formation of blood vessels from pre-existing capillaries. Tumor cells are able to grow around an existing vessel and at least initially, do not need to induce angiogenesis for adequate vascularization[16].

Furthermore, circulating endothelial precursors, angioblast-like cells derived from bone marrow but reported to be present in the adult circulation, have recently been suggested contributing to tumor-derived blood vessels[17].

Tumor induced vessels form a conduit for the delivery of metabolites, but ultrastructurally they are abnormal. Many lack functional pericytes, they are dilated and convoluted, and they are exceptionally permeant due to the presence of the fenestrae and transcellular holes and lack of a complete basement membrane. Furthermore, tumor vessel walls may be made up of both endothelial cells and tumor cells[18].

Factors that regulate tumor angiogenesis are – VEGF, FGF, Heparanase, Ang 2, IL-8, MMP-2, Transforming growth factor-β[19].

In the majority of cancers, highly vascularized tumors showed a poor prognosis and the influence of tumor angiogenesis is proved to be independent of the conventional prognostic indicators.

Oral squamous cell carcinoma arises de novo or from pre-existing lesions. The progression of oral epithelial dysplastic lesions to squamous cell carcinoma is characterized by an increase in neo- vascularization in the sub-epithelial lamina propria and can be considered as an indicator of malignant transformation[20].

Angiogenesis can also be triggered by hypoxia resulting from increasing distance of the growing tumour to the capillaries. Tumour angiogenesis is a complex event, which mediated by angiogenic factors released by cancer cells or by host immune cells[21].

It has been reported that that endothelial cells of tumour associated neovasculature proliferate 20 to 2,000 times more rapidly than the endothelial cells of normal tissue[22].

Angiogenesis is pivotal in the migration, growth, and differentiation of endothelial cells in normal physiological conditions. In various types of tumour microenvironments, dysregulated angiogenesis plays an important role in supplying oxygen and nutrients to cancerous cells, leading to tumour size growth. Vascularization is essential for tumor growth and metastasis. Preceding tumour growth beyond a certain size, hypoxic conditions initiate spontaneous secretion of angiogenic stimulators, which is an important step for transition of a benign tumour to a malignant state[23].

Insufficient supply of blood, causes tumor cells to undergo apoptosis and necrosis. Distinct tissue distribution and known functional integration with TGF-β system, highlights the fact that endoglin is involved in angiogenesis. High levels of endoglin expression on actively proliferating tumoral endothelial cells on the luminal surface allows for immunoscintigraphy of tumors, which can be used for the diagnostic purposes[24].

Angiogenic markers should ideally detect quality and quantity of newborn vessels[25].

The ability to visualize and quantify tumor angiogenesis may not only allow antiangiogenic treatment monitoring in patients with cancer but may also be an appropriate approach for screening and detection of cancer at an early, still curable stage, just after the angiogenic switch has occurred in the tumor progression. Several molecular angiogenic markers overexpressed in tumor are- αvβ3 Integrin, endoglin, and vascular endothelial growth factor (VEGF) receptor (VEGFR) 2, are the best-characterized molecular markers of tumor angiogenesis[26].

The proposition that CD105 may be associated with tumour angiogenesis developed from fact that CD105 was strongly up-regulated in the endothelium of various tumour tissues compared with that in the normal tissue[22].

The number of blood vessels is more in a growing OPMD, when compared to healthy oral mucosa, and it further increases in an enlarging OSCC[27].

Previous studies done by **Shieh YS et al**[28] and **Iamaroon A et al**[29] have shown that vascularity increased progressively from normal mucosa through dysplasia to carcinoma, which suggests that disease progression in the oral mucosa is accompanied by angiogenesis.

Shivakumar Madappa Shivamallappa[30] and **Macluskey et al**[31] their study implied the fact that, in the development of OSCC angiogenic phenotypic change occurs in carcinomas rather than pre-cancerous stage. Previous studies have shown that vascularization increased with an increasing degree of dysplasia by microvascular volume[29,31].

Mast cells have surface receptors, which when stimulated may release proangiogenesis factors, such as vascular endothelial growth factor (VEGF), basic

fibroblastic growth factor (bFGF), transforming growth factors, IL-8, matrix metalloproteinase, tryptase, and chymase that participate in the neo angiogenesis or vascular formation process.

It has been hypothesized that the angiogenic phenotype may result from the production of these growth factors by the tumor cells and/or the down-regulation of negative modulators, like thrombospondin-1 (TSP-1) in tissues with dormant vasculature[32].

MAST CELLS

Cancer is a progressive disease, occurring in a series of well-defined steps. Initiated cells require alterations rendering them self-sufficient for growth, insensitive to growth-inhibitory signals, resistant to programs of terminal differentiation, or apoptosis, as well as endowing them with unlimited self-renewal capacity, the ability to direct sustained angiogenesis, and the ability to invade and thrive in ectopic tissue environments[33].

Mast cells (MC) are bone marrow-derived tissue-homing leukocytes, first described by Paul Ehrlich. They are highly versatile cells playing important role in large spectrum of biological settings, including inflammation, angiogenesis, tissue repair, tissue remodeling and cancer[34].

They are component of the immune system playing an important role in host defence. They reside in all the vascularised tissues, especially in all the connective tissues and mucosal surfaces of the human body[35].

Stem Cell Factor plays an important role in the survival of mast cells because it stimulate8 directional motility of mucosal and connective tissue type of mast cells[36].

Many studies have demonstrated tumour growth induction secondary to activation of mast cell. Diverse mechanisms such as the release of pro-angiogenic inducers and activation of CD30 by mast cells have been reported[37].

Mast Cells are heterogeneous in shape, varying from round, oval, or spindle-shaped, about 12 microns (diameter) with 50 - 100 packed granules. It is often difficult to distinguish fibroblasts from these cells in routine hematoxylin and eosin (H&E) stain owing to its similar staining characteristics. Hence, a selective metachromatic stain of 1% toluidine blue was used first in 1856 by William Henry Perkin (British chemist) who illustrated purplish-red MCs and their nuclei appearing sky blue in color[38].

Mast cells are easily also recognized in light microscopy by toluidine blue staining because of metachromatic granules that fill the cytoplasm. In normal oral tissues, mast cells have been found to be present in the connective tissue of gingiva, tongue and lining mucosa, also reported in normal periodontal ligament and pulp although in very low densities. The numerous cytoplasmic granules in the mast cells bind to basic dyes such as toluidine blue and show metachromatic staining properties[39].

Under physiological conditions, they are particularly prominent near capillaries and also lymphatic channels. In many inflammatory disorders, they are characterized by profound vascular remodelling, and the infiltrate exhibits numerous mast cells which show structural features of degranulating elements. In various tumors, mast cells appear at the edges of invasive tumors, facilitate angiogenesis by releasing preformed mediators or by triggering proteolytic release of extracellular matrix bound angiogenic compounds[40].

Rakesh et al[39] in their study reported that mast cells and their regulatory role in angiogenesis and inflammation by the release of mediators may aid in tumor

progression, facilitating the transformation of oral leukoplakia into invasive carcinoma.

Ankle MR, Kale AD, Charantimath S[41] in their study found a significant correlation between MCD and MVD in OSCC, concluding that MCs may promote tumor progression by regulating angiogenesis.

Metachromatic dyes such as toluidine blue, causes more clearer visual detection of mast cells, hence a reliable method to be used for the same.

TOLUDINE BLUE STAIN

Chairside techniques that are assessed to improve earlier detection and diagnosis of oral malignancy include toluidine blue, vizilite plus with touidine blue, velscope brush biopsy, etc. Toluidine blue is a cationic metachromatic dye that may selectively bind to free anionic groups such as sulfate, phosphate, and carboxylate radicals of large molecules. It has been used for decades as an aid to identify mucosal abnormalities in the oral cavity. Toluidine blue, also known by its chemical name tolonium chloride, is a basic metachromatic dye as well as a supravital stain that is known for its property of differentially staining malignant neoplasm but not normal epithelium[42].

Mast cells are difficult to differentiate with Haematoxylin and Eosin, but under different metachromatic staining, mast cell granules will appear purplish blue with nuclei- sky blue in toluidine blue stain[43].

MAST CELLS IN OSCC

Mast cells secrete various proteolytic enzymes which degrade the extracellular matrix and create space for developing blood vessels[44].

Among the host immune cells, mast cells have been implicated in tumour progression as it promote angiogenesis. They are a source of several proangiogenic and angiogenic factors such as histamine, heparin, chymase, basic fibroblastic growth factor, vascular endothelial growth factor and transforming growth factor[45].

Microvessel density can be used as a method for assessment of angiogenesis. The technique involves the immunohistochemical staining of endothelial cells of the capillaries by using monoclonal antibodies against like CD105[46].

Biviji AT[47] in their study showed an increase in the number of mast cells/unit microscopic field in oral leukoplakia (which represents increase in the thickness of the epithelium, its keratinization without dysplasia) compared to normal mucosa. They concluded that pharmacologically active agents in the mast cells might contribute to inflammatory reaction, which is seen in leukoplakia.

Flynn E, Schwartz j, Shklar G[48] reported direct co-relation between sequential mast cell infiltration, activation and distinct stages of hyperkeratosis, dysplasia, carcinoma in-situ in the oral cavity and implicated role of mast cells in configuring the angiogenic phenotype in premalignant lesions.

A hypothesis was suggested that hypoxia might induce tumour cells to release the angiogenic factors, which in turn could attract mast cells to migrate into the hypoxic areas of the tumour and produce stimulating factors that help in further angiogenesis. Mast cells are also an important source of several pro-angiogenic factors, such as-histamine, heparin, chymase, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and transforming growth factor-β (TGF-β) etc[21].

These angiogenic factors either directly promote angiogenesis by stimulating the migration and/or proliferation of endothelial cells or indirectly through degradation of the extracellular matrix[45].

Hegde Veda and Marla Vinay[44] in their study concluded that tumours that are rapidly growing may have a high nutritive demand that is provided by the vasculature, and when the epithelium is altered as in oral epithelial dysplasia, recruitment of inflammatory cells is noted. The inflammatory and mast cells that migrate to areas of altered epithelium may stimulate angiogenesis by secreting proangiogenic and angiogenic components, either directly or indirectly prior to invasion. Once invasion is established as in oral squamous cell carcinoma, the role of mast cells is probably shifted from angiogenesis to further promoting invasion.

Increased mast cells are also been associated with squamous cell carcinomas. A correlation between mast cell numbers and poor outcome was reported by **Iamaroon A et al**[29] on oral squamous cell carcinoma.

As the neoplastic sequence progresses, angiogenic growth factor gene expression is upregulated in cancer cells and this is the progression to the second cancer phase where tumor cells directly control their angiogenic phenotype instead of manipulating inflammatory cells to indirectly affect neovascularization[49].

Belgaumi UI et al[50] in their study we found a decrease in MC count associated with an advanced histologic grade of OSCC, hence poorer prognosis, thus favouring the protective role of mast cells in oral squamous cell carcinoma.

Studies have suggested that the mast cell population is significantly more in oral squamous cell carcinomas than in oral epithelial dysplasia[51].

Recent studies showed that mast cell in perilesional and intra tumoural area of oral squamous cell carcinoma expresses CD105, VEGF, VEGFR1, and VEGFR2 and showed positive correlation with angiogenic activity of the tumour[52].

Mast cells play a role in a variety of physiological and pathological processes, like cancer. They act as gate keepers of the immune system and in turn respond to many signalling pathways, thus contributing carcinogenesis and metastasis. Increased expression of c-Kit and stem cell factor, which is necessary for migration, maturation, and survival of mast cells, has been observed in tumours[53].

Tumour promotion by mast cells is considered to be due to its angiogenic ability. Evidence of degranulation and angiogenesis due to mast cells in pre-malignancy has also been observed in experimental studies[54].

Mast cells can also modulate haemostasis and blood perfusion in tumours by production of heparin[55].

The other possible methods in which mast cells promote tumours is by mitogenic ability, deletion of tumour suppressor genes, and activation of certain oncogenes via the c-kit locus[56].

Secretion of proteases, which contributes to extracellular matrix degradation, formation of vascular tube, release of trapped angiogenic factors, promotion of metastasis is possibly another mechanism by which mast cells promote tumours. Mast cells have been found to have both direct mitogenic activities on tumour cells as well as an indirect effect on the microenvironment by promoting invasive potential. (Brought about mainly by heparin and TNF-α factor)[57].

Main factors released are heparin, histamine, chymase, tryptase, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and TGF-β.

Tryptase helps in the process of neovascularization, increasing the number of fibroblasts and matrix reorganisations with type α1 procollagen mRNA. Cytokines especially IL-8 produced by mast cells induce matrix metalloproteinase 2 (MMP-2), thereby facilitating endothelial cell migration assisting angiogenesis[58].

Narayan KV et al[59] conducted a comparative study to assess mast cell count in oral squamous cell carcinoma (OSCC) at different histological grades using toluidine blue stain. The findings revealed that mast cell density increased as the tumor grade advanced, with high-grade OSCC showing the highest mast cell counts. This suggests that mast cells may contribute to tumor progression by supporting angiogenesis and modulating the immune response. The study concluded that higher mast cell counts in advanced tumor grades are associated with tumor aggressiveness and poor prognosis.

Shrestha A, Keshwar S, Raut T[60] evaluated mast cell density in oral potentially malignant disorders (OPMDs) and oral squamous cell carcinoma (OSCC) to understand their role in tumor progression. The study found that mast cell infiltration was significantly higher in OSCC tissues compared to OPMDs, especially in areas of tumor invasion. The authors highlighted that mast cells, by releasing pro-inflammatory mediators, may play a role in tumor progression by enhancing angiogenesis and immune suppression. Thus, the study concluded that higher mast cell counts in OSCC may indicate tumor aggressiveness, and targeting these cells could provide a therapeutic approach for preventing malignant transformation and metastasis in oral cancers.

Cai XJ et al[61] investigated the role of mast cell infiltration and its subtypes in the malignant transformation of oral precancerous lesions and progression of oral cancer. The study analyzed tissue samples to assess mast cell density and subtype distribution

in different stages of oral carcinogenesis, and the results revealed a significant increase in mast cell infiltration in oral precancerous lesions that progressed to malignancy, with specific mast cell subtypes promoting tumor growth, angiogenesis, and immunosuppression. Their findings also suggested that mast cells play a crucial role in the tumor microenvironment, facilitating the transition from precancerous to cancerous states and influencing tumor aggressiveness. The authors focused on the potential of targeting mast cell activity as a therapeutic strategy for preventing malignant transformation and controlling oral cancer progression.

Khan Y, Rehani S, Sharma M[62] investigated the role of mast cells in angiogenesis across various histopathological grades of oral squamous cell carcinoma (OSCC) using histochemical techniques. The findings of the study showed a significant increase in mast cell count in higher grades of OSCC, particularly in poorly differentiated carcinomas, where aggressive tumor behavior is observed. The study revealed that mast cells may contribute to neovascularization, supporting tumor growth, invasion, and metastasis.

CD105

Endoglin, a 180 kDa transmembrane protein, which occurs in two isoforms (L and S) because of alternate splicing of the transcript, and it shows higher expression in proliferating endothelial cells. It The CD105 expression in tumor and normal endothelium are highly correlated[63].

Gadbail AR et al[64] conducted study to determine mutations in the **ENG** gene, which encodes CD105, associated with hereditary haemorrhagic telangiectasia type 1 (HHT1), a genetic disorder which is characterized by vascular malformations and recurrent bleeding. Given its dual role in promoting vascular integrity and being

implicated in pathological angiogenesis, CD105 is a critical for understanding the vascular biology and developing therapeutic strategies.

This auxiliary TGF- β receptor transmembrane glycoprotein is expressed on activated endothelial cells participating in neoangiogenesis. Evaluation of MVD using endoglin can be an effective tool in quantifcation of neovascularization in carcinomas and also determines the metastatic potential ofthe tumor and its prognosis[65].

Human endoglin is a 633 amino acid, 180 kDa homodimeric disulfide-linked hypoxia-inducible transmembrane glycoprotein and it contains a large extracellular domain, a hydrophobic transmembrane domain, and a short intracellular domain. The extracellular domain contains an Arg-Gly-Asp (RGD) tripeptide, and four N-linked glycosylation sites and a region of O-linked glycosylation[66,67].

The intracellular domain contains many serine and threonine residues, some of which are phosphorylation sites[68].

The endoglin gene is 40 kb long and it is located on human chromosome 9q34[69].

Gene transcription results in a mRNA product with a length of 3.4 kb and 14 exons; exons 1 to 12 encode the extracellular domain, and the exon 13 encodes the transmembrane domain, and the exon 14 encodes the cytoplasmic domain. Endoglin was assigned the cluster of differentiation number 105 at the Fifth International Workshop on Human Leukocyte Differentiation Antigens and thus is also known as CD105[70].

Endothelial cells in normal quiescent endothelium are stable, have a very low turnover rate, with a doubling time of more than 1,000 days. Angiogenic endothelium, in contrast, has a rapid turnover, has been termed ''activated''. One potential marker

of this activated endothelium is endoglin. In several malignancies, endoglin is found on the peritumoral and intratumoral vessels[71].

TGF-β signaling in endothelial cells can mediate variety of processes, like proliferation, migration, and adhesion. TGF-β is expressed in endothelial cells of healing wounds, embryos, infected tissues, psoriatic skin, inflamed synovial arthritis, and solid tumors.

Low doses of TGF-h in vitro (0.25-0.70 ng/mL) stimulate endothelial cell proliferation and migration, whereas higher doses inhibit them, which endothelial cell quiescence[72].

Stimulatory phenotype in endothelial cells is mediated by ALK1-dominated signaling, and endoglin expression is necessary for the activation of this pathway. ALK1 activation causes increase in endothelial cell proliferation and migration as well as transcription of the proangiogenic genes Id1, interleukin- 1 receptor-like 1, and endoglin itself[73].

In endoglin-expressing cells, endoglin is included as a dimer in this receptor complex, and TbetaR-II then phosphorylates either ALK1 or ALK5 in a highly conserved glycine/serine-rich cytoplasmic domain, causing a conformational change in the receptor. Signaling is propagated to the nucleus via phosphorylation of Smad proteins, which act as transcriptional coactivators or corepressors.

In endothelial cells, endoglin expression is up-regulated by hypoxia, TGF-h stimulation, and irradiation in vitro, whereas endoglin is down-regulated by tumor necrosis factor-alpha[74].

This (CD105) transmembrane phosphorylated glycoprotein, a fundamental constituent of the TGF-β receptor signaling pathway. The proliferation and differentiation of cells

is modulated by this pleiotropic cytokine, which is fundamental for neoangiogenesis and vascular growth. Multiplying endothelial cells show upregulation of CD105, and so is considered as a principal marker of proliferation of endothelial cells of newly formed blood vessels that is neoangiogenesis[24].

According to **Nikolaos A Dallas et al**[71], expression of CD105 expression in normal human tissue is shown in Table 2:

Table 2: Endoglin (CD105) expression in normal human tissues

Location	Cell type
Cardiovascular system	Endothelial cells Vascular smooth muscle cells
Hematologic system	Bone marrow stromal fibroblasts Progenitor B cells Erythroid precursors Circulating $CD34^+$ cells
Reticuloendothelial system	Interstitial macrophages in splenic red pulp Activated monocytes Differentiated macrophages Follicular dendritic cells in lymphoid organs
Genitourinary system	Kidney extraglomerular interstitial cells Kidney glomerular mesangial cells Seminiferous tubule basal cells
Integumentary system	Melanocytes
Embryo	Syncytiotrophoblasts of placenta Embryonic heart mesenchymal cells

CD105 in OSCC

Several previous reports had shown that CD105 expression was related to metastasis, whereas only few studies investigating CD105 expression in head and neck regions have been reported. Endoglin CD 105 expression was studied by many workers in various oral lesions like, oral submucous fibrosis, angiogenic squamous dysplasia-like phenomenon in oral epithelial precursor lesions.

According to **Raunak Kumar Das et al**[74], expression of Endoglin CD 105 in normal oral mucosa was completely absent. The expression of CD 105 in epithelial dysplasia, in premalignant lesions, though variable, but is expressed faintly in oral submucous fibrosis without dysplasia, it became remarkable in dysplastic conditions, with maximum presence in severe oral submucous fibrosis condition, which indicates that CD105 expression, a marker for tumour vasculature, was upregulated in dysplastic conditions.

Study on quantification of MVD with CD105 was done by **Maharudrappa Basanker, Shashikant SR, Satish BNVS**[75] which revealed that highest density was present in tumour specimens, than in normal healthy mucosa and dysplastic epithelium.

The expression of CD105 observed by different researchers in OSCC was significantly higher i.e., to the extent of 75% of cases[76].

A Bhargav Kumar Gudipati et al[77] in their study showed that Mean Vascular Density (MVD) was not significant between three different groups of OSCC but there was significant difference between normal mucosa and different grades of dysplasia as when compared to OSCC. Also, WDSCC showed high amount of MVD compared

to all the groups. Role of endoglin determines the metastatic potential of the tumor and its prognosis, thus, could be considered as a potential target of therapy.

Patil BR et al[78] conducted a study to compare the immunohistochemical expression of vascular endothelial growth factor (VEGF) and CD105 in oral squamous cell carcinoma. They found both VEGF and CD105 exhibited significantly higher expression levels in OSCC tissues compared to normal oral mucosa indicating their involvement of angiogenesis of OSCC. Their results showed that increased CD105 expression was associated with more aggressive tumor features, such as higher tumor grade, greater depth of invasion, and the presence of lymph node metastasis. Also, a higher expression of CD105 was correlated with poorer clinical outcomes, including shorter survival and higher recurrence rates. The study highlighted that while both VEGF and CD105 contribute to angiogenesis, CD105 had a stronger correlation with the prognosis of OSCC. Their finding suggested that CD105 could be a useful prognostic marker for assessing the aggressive behavior OSCC and could potentially serve as a target for therapeutic strategies aimed at inhibiting angiogenesis in OSCC.

Niva Mahapatra et al[65] conducted study on the expression of endoglin (CD105) in oral squamous cell carcinoma (OSCC) and its potential role in tumor angiogenesis. Results of the study showed a significant increase in CD105 expression in the tumor vasculature compared to the adjacent normal tissue, suggesting that CD105 plays a crucial role in the angiogenesis process in OSCC. The study also investigated the correlation between CD105 expression and various.

MATERIALS AND METHODS

MATERIALS AND METHOD

This retrospective comparative study was conducted using the archival slides of pre-diagnosed cases from the Department of Oral Pathology and Microbiology, Babu Banarasi Das College of Dental Sciences, BBD University, Lucknow.

Study Sample: 10 cases each of well, moderately and poorly differentiated squamous carcinomas and pyogenic granuloma as control were immunohistochemically analysed for assessment MVD using expression of Endoglin (CD-105) and mast cell density using toluidine blue stain

Pyogenic Granuloma (PG) was used as control because it is an inflammatory condition which is known to have increased number of blood vessels than the normal oral mucosa.

Eligibility Criteria:

Inclusion criteria:

- Cases previously diagnosed and reported as well, moderately or poorly differentiated squamous cell carcinoma.
- Cases previously diagnosed and reported as pyogenic granuloma.

Exclusion Criteria:

- OSCC's with inadequate tissue depth
- Carcinomas other than conventional oral squamous cell carcinoma
- Recurrence and metastatic tumors of the oral cavity

Sampling Method: Random Sampling

Methodology:

One section each were taken to stain with Hematoxylin & Eosin, Toluidine Blue and CD105 respectively, thereby divided into 3 groups :

Group1 (10samples) : Well Diffeentiated Squamous Cell Carcinoma

Group2 (10samples) : Moderately Differentiated Squamous Cell Carcinoma

Group 3 (10 samples) : Poorly Differentiated Sqamous Cell Carcinoma

Control sections (10 samples): Pyogenic Granuloma

A total of 40 formalin-fixed, paraffin-embedded tissue samples were collected and mounted on slides by consecutive, non-probability sampling technique.

Methodology for CD105

One section of 3-4 μm thickness was taken from each block. CD 105 antibody (BIOGENEX) was applied onto sections on Poly-L-lysine coated slides. Antigen retrieval was done by transferring the slides to citrate buffer of pH 6.2 and autoclaved at 15 lbs pressure for 30 min. The slides were treated with 3% hydrogen peroxidase for 10 min to quench endogenous peroxidase activity. Prediluted primary antibody, namely mouse monoclonal CD105 antibody (BioGenex), were added to the sections and incubated for 60 min in a hydrated chamber. The sections were washed with tris buffer for 2–3 min and a super-enhancer reagent was added and incubated for 30 min. The sections were washed with tris buffer for 2–3 min and incubated for 60 min with a secondary antigen that is polymer-Horseradish peroxidase immunohistochemistry, 3,3′ (HRP IHC) detection system. Chromogen Diaminobenzidine (DAB) were added and incubated for 20 min, then washed with tris buffer and counterstained with hematoxylin. Specimens of pyogenic granuloma known to express CD105 positive cells were used as positive control. CD105 staining is localized to cell membranes of

the endothelium of blood vessels. Prior to assessment, slides were dehydrated with immersion in 70%, 90% and 100% ethanol for 5 min each respectively, followed by Xylene immersion (5 min) and mounted using DPX[65].

Scoring criteria:

Stained sections were interpreted microscopically under the penta head microscope (Olympus Magnus MLXi Plus). Slides were first be screened using a low-magnification objective lens. The areas with the highest number of positively stained vessels (hot spots) were identified and selected. At x400 magnification ($0.0625mm^2$), the microvessels (recognized by brown cytoplasmic staining by CD105) were counted using oculometer grid with 100 subdivisions. This was done for 05 fields and average of the 05 fields were taken. The criteria adopted by Weidner et al[79], were used and only strong positively stain endothelial cell or cell cluster, which is easily separated from adjacent micro vessel and other connective tissue elements were considered as a distinct countable micro vessel. Two observers simultaneously evaluated all the slides by Penta head microscope (Olympus Magnus MLXi Plus). Each micro vessel which was agreed by both observers were included in the count. Micro vessel density is defined as the number of CD105 (Endoglin) stained vessels per mm^2.

Formula for calculation of microvessel density= (Number of microvessels in x400 grid field)/Area of ocular grid ($0.0625mm^2$)[80].

Methodology for Toludine Blue[81]

Toluidine Blue staining was done for all grades of OSCC and mast cells were assessed.

Tissues fixed in 10% Formalin were cut at 5 um paraffin sections.

Solutions and Reagents:

Toluidine Blue Stock Solution:

Toluidine blue O (Sigma)----------1 g

70% alcohol----------100 ml

Mixed to dissolve.

Sodium Chloride (1%):

Sodium chloride----------0.5 g

Distilled water----------50 ml

Mixed to dissolve (made this solution fresh each time). Adjust pH to 2.0~2.5 using glacial

acetic acid or HCl.

Toluidine Blue Working Solution (pH 2.0~2.5):

Toluidine blue stock solution----------5 ml

1% Sodium chloride, pH 2.3----------45 ml

Mixed well. The pH should be around 2.3 and less than 2.5

Made this solution fresh and discarded after use. pH higher than 2.5 will make staining less contrast.

Procedure:

1. Deparaffinize and hydrate sections to distilled water.
2. Stain sections in toluidine blue working solution for 2-3 minutes.
3. Wash in distilled water, 3 changes.
4. Dehydrate quickly through 95% and 2 changes of 100% alcohol (10 dips each since stain fades quickly in alcohol).
5. Clear in xylene or xylene substitute, 2 changes, 3 minutes each.

6. Coverslip with resinous mounting medium.

Identification & Scoring criteria for Mast cells:

Mast cells----------violet/red purple.

Background----------blue.

Scoring criteria:

Stained sections were interpreted microscopically under the penta head microscope (Olympus Magnus MLXi Plus). Slides were first be screened using a low-magnification objective lens. The areas with the highest number of positively stained vessels (hot spots) were identified and selected. At x400 magnification ($0.0625mm^2$), the mast cells (recognized by blue cytoplasmic staining by Toluidine blue) were counted using oculometer grid with 100 subdivisions. This was done for 05 fields and average of the 05 fields were taken. The criteria adopted by Weidner et al[79], was used and only strongly stained mast cells were considered. Two observers simultaneously evaluated all the slides by penta head microscope (Olympus Magnus MLXi Plus) . Each mast cell which was agreed by both operators were included in the count. Mast cell density is defined as the number of mast cells per mm^2.

Formula for calculation of mast cell density= (Number of mast cells in x400 grid field)/Area of ocular grid ($0.0625mm^2$)[80].

LEGENDS

LEGENDS

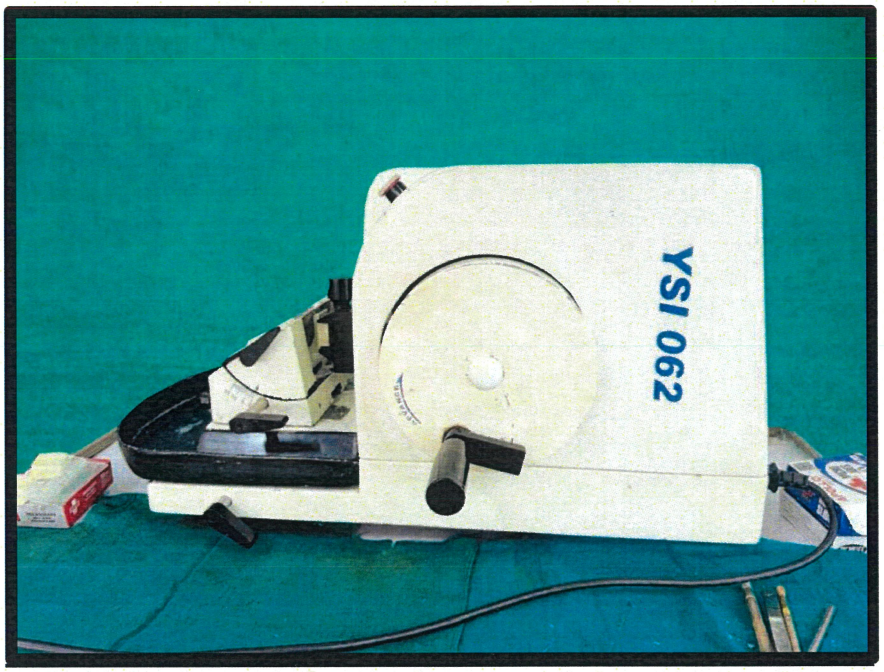


Figure 1: Semi Automatic Rotary Microtome (Yorco YSI 062)



Figure 2: Decloaking Chamber

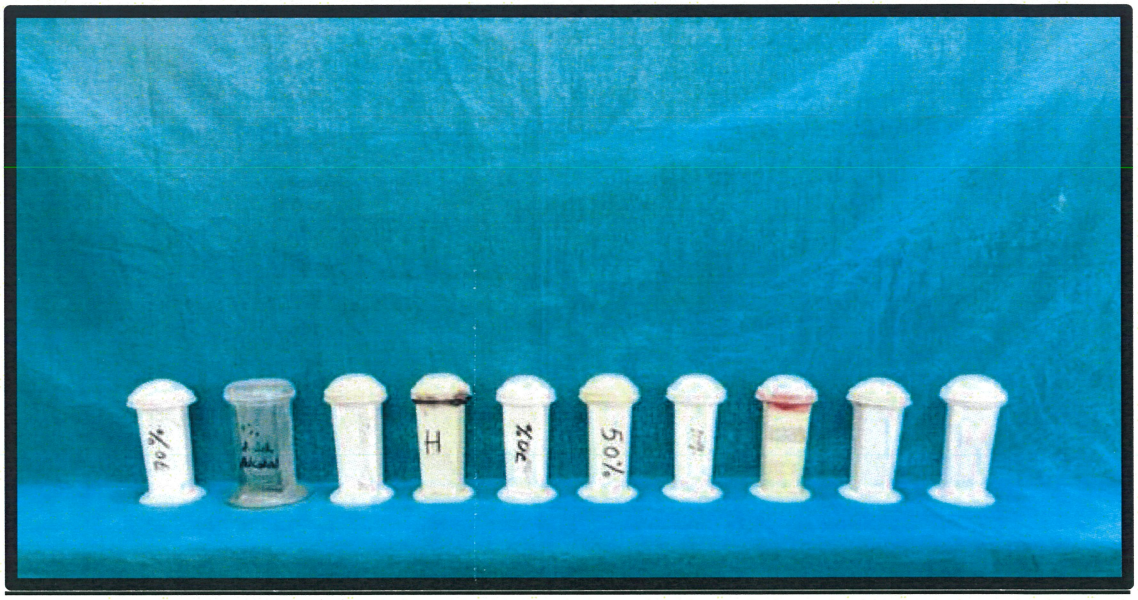


Figure 3: Reagents used for Hematoxylin & Eosin staining



Figure 4: CD105 antibody (BioGenex) used for Immunohistochemical staining

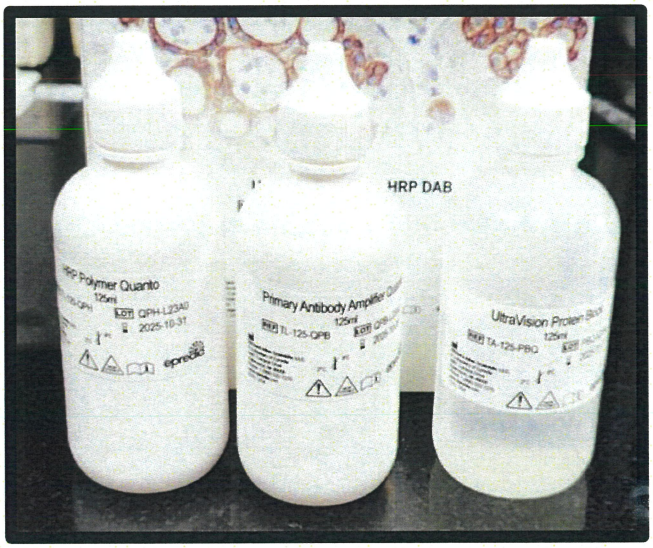


Figure 5: Other reagents used for Immunohistochemical staining



Figure 6: Tris buffer



Figure 7: Ultra Vision HRP DAB

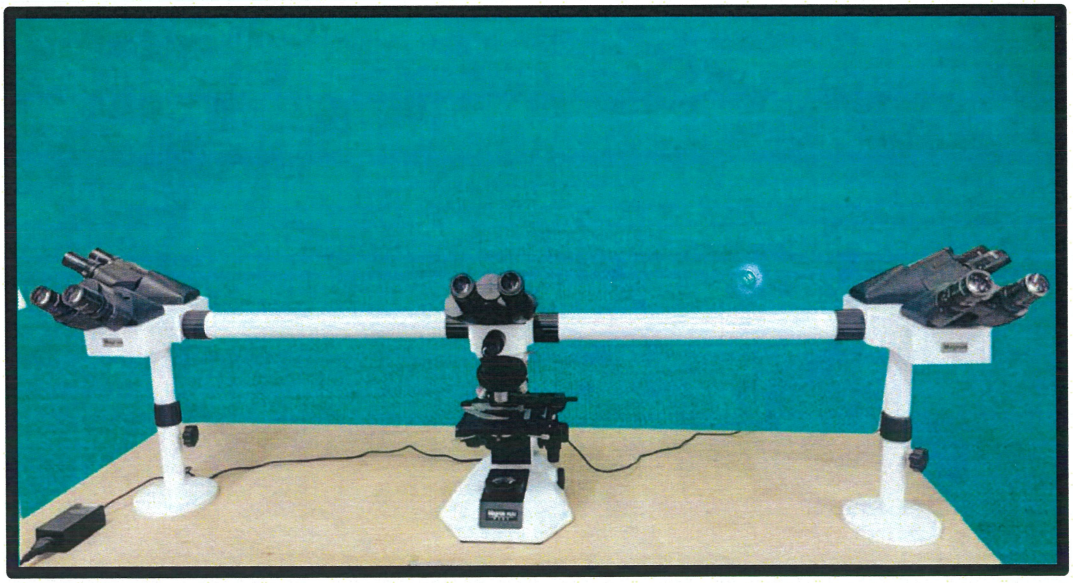


Figure 8: Pentahead microscope (Olympus Magnus MLXi Plus)

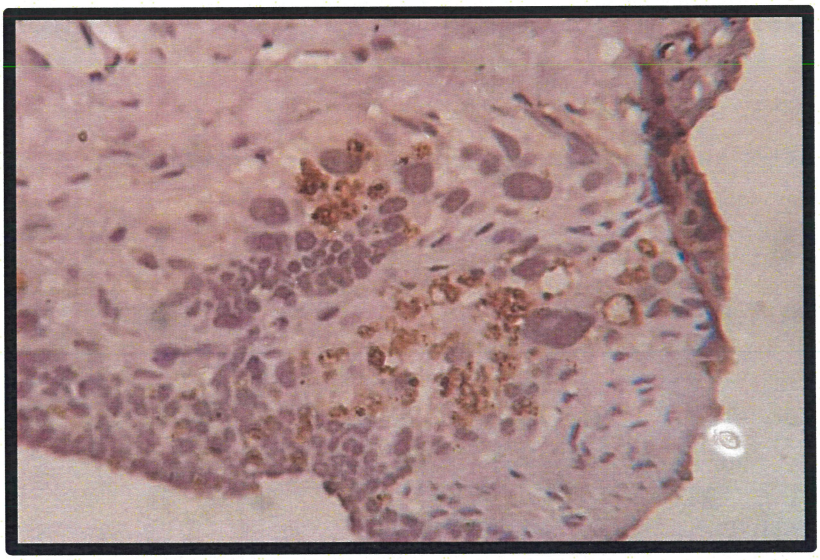


Figure 9 : Control Section for CD105

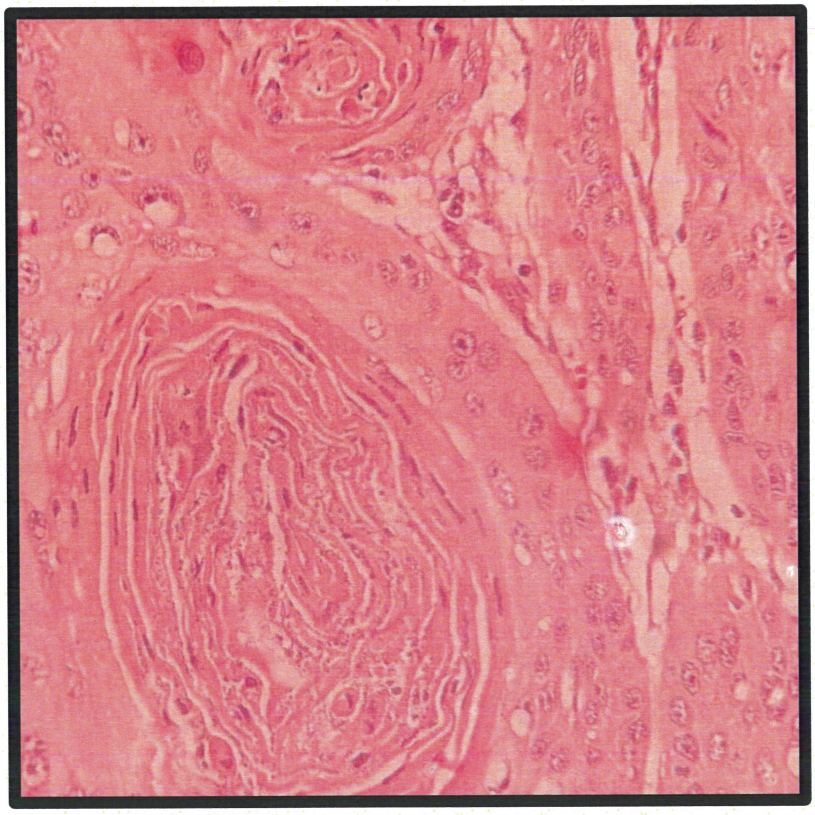


Figure 10 : Photomicrograph showing H& E stained section well differentiated squamous cell carcinoma (x400)

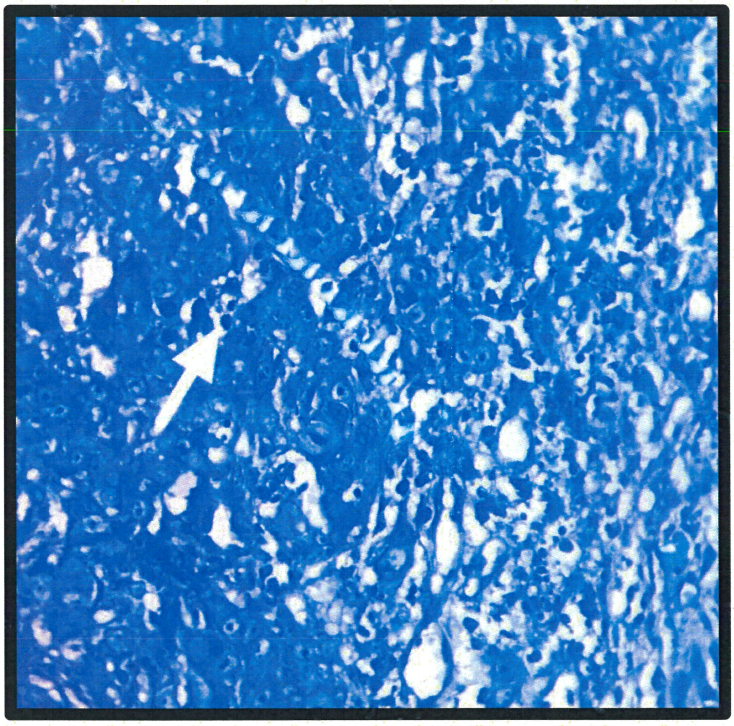


Figure 11 : Photomicrograph showing mast cells in well differentiated squamous cell carcinoma (Toluidine Blue Stain) (x400)

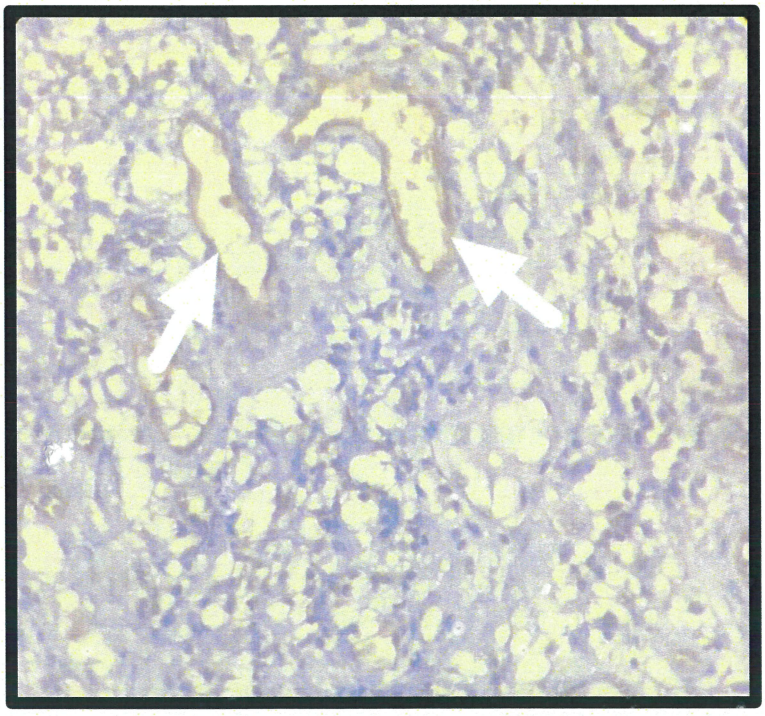


Figure 12 : Photomicrograph showing CD105 positive endothelial cells in well differentiated squamous cell carcinoma (x400)

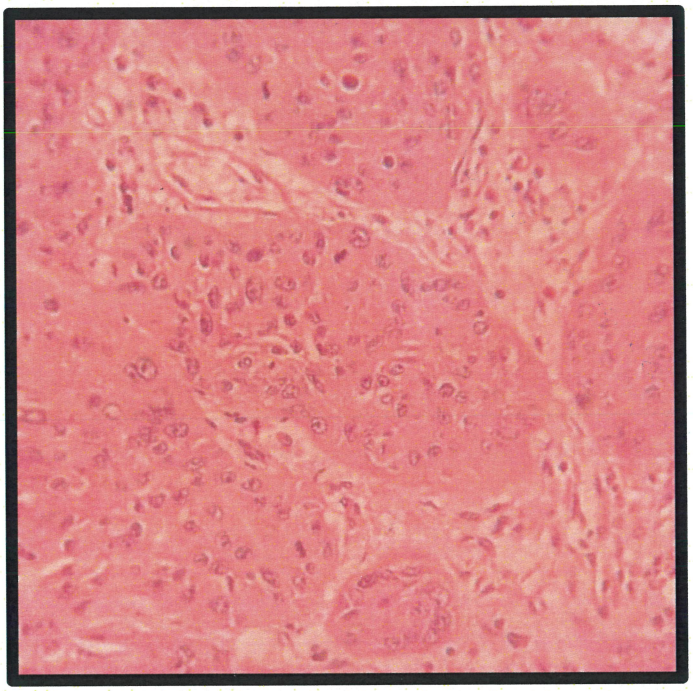


Figure 13 : Photomicrograph showing showing H& E stained section of moderately differentiated squamous cell carcinoma (x400)

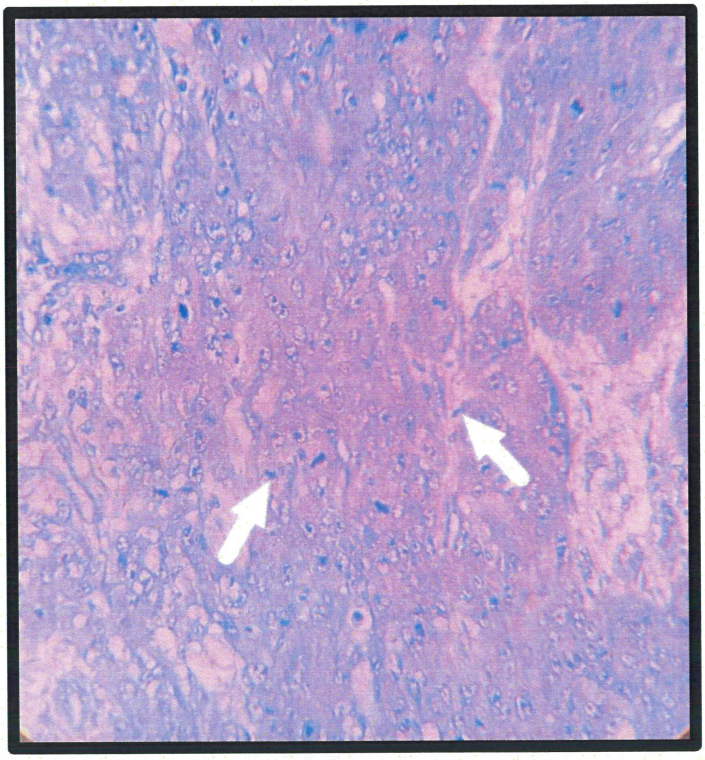


Figure 14 : Photomicrograph showing mast cells in moderately differentiated squamous cell carcinoma (Toluidine Blue Stain) (x400)

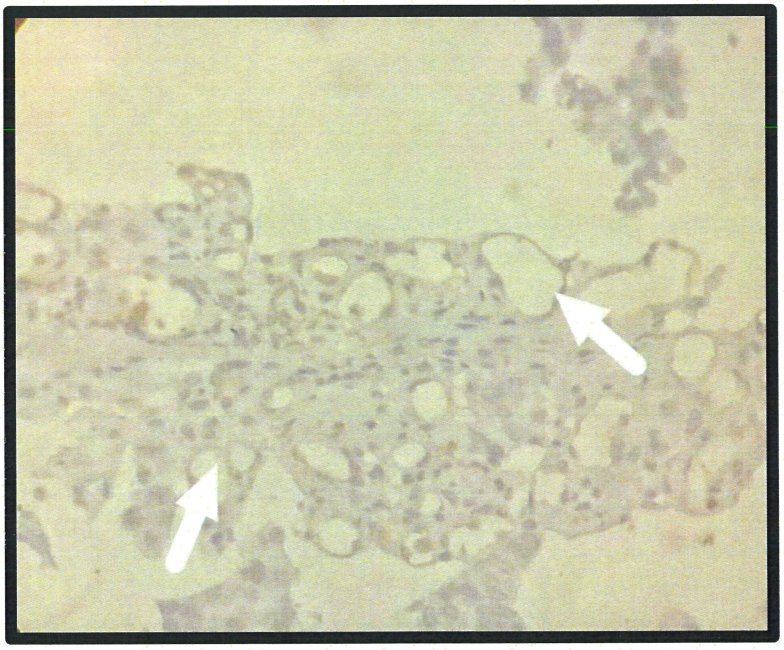


Figure 15 : Photomicrograph showing CD105 positive endothelial cells in moderately differentiated squamous cell carcinoma (x400)

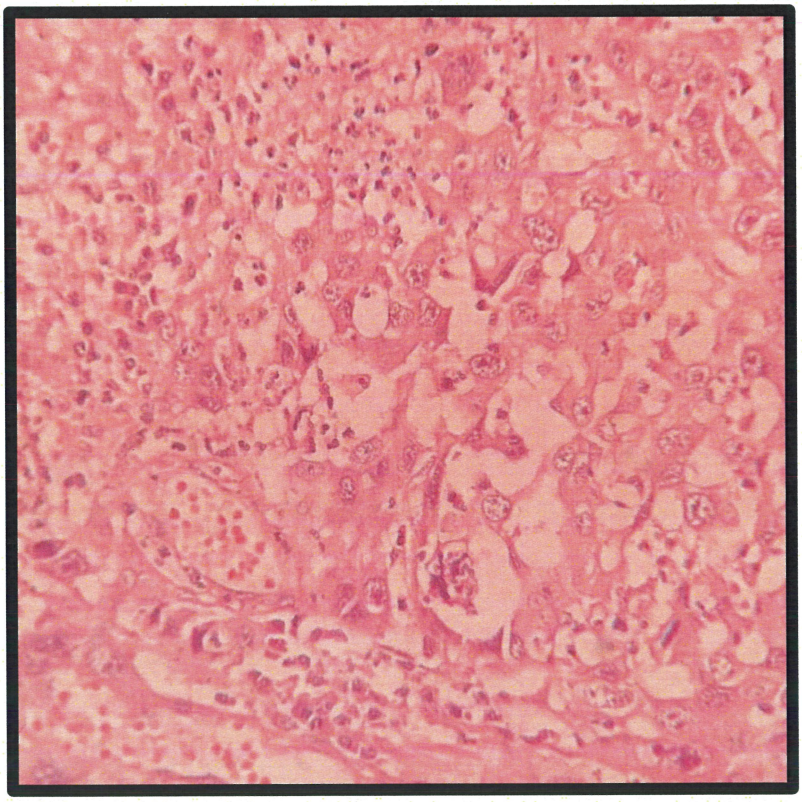


Figure 16 : Photomicrograph showing H& E stained section of Poorly differentiated squamous cell carcinoma (x400)

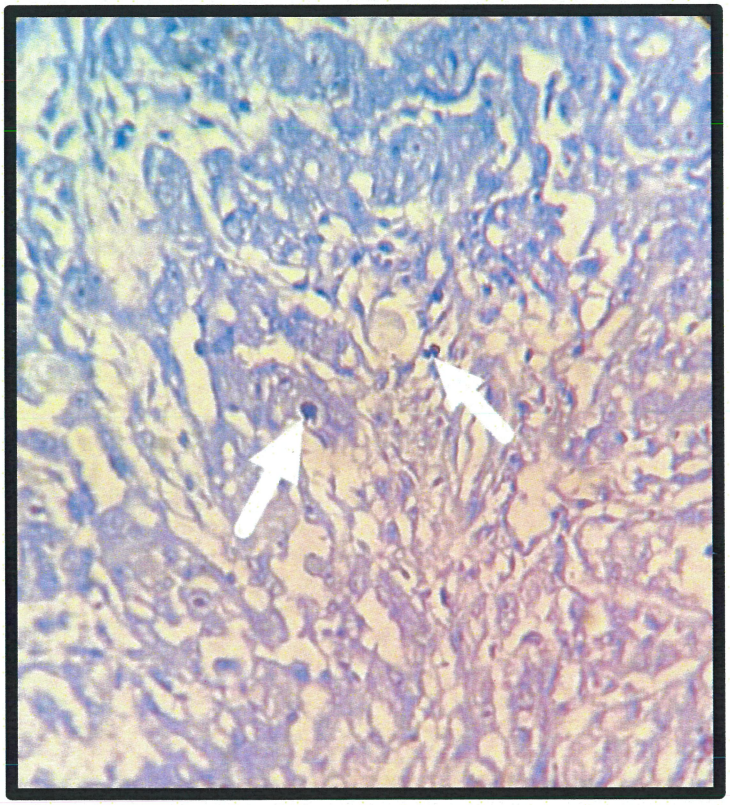


Figure 17 : Photomicrograph showing mast cells in Poorly differentiated squamous cell carcinoma (Toluidine Blue Stain) (x400)

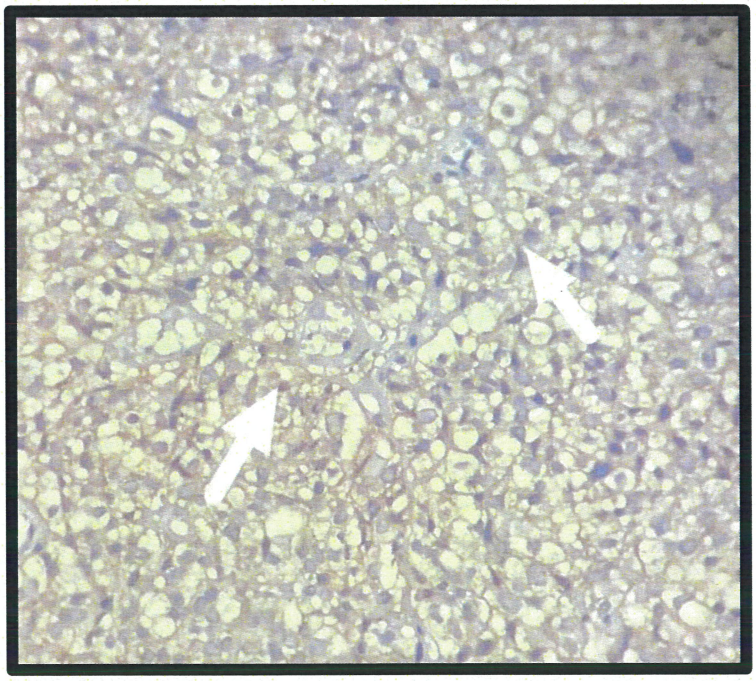


Figure 18 : Photomicrograph showing CD105 positive endothelial cells in Poorly differentiated squamous cell carcinoma (x400)

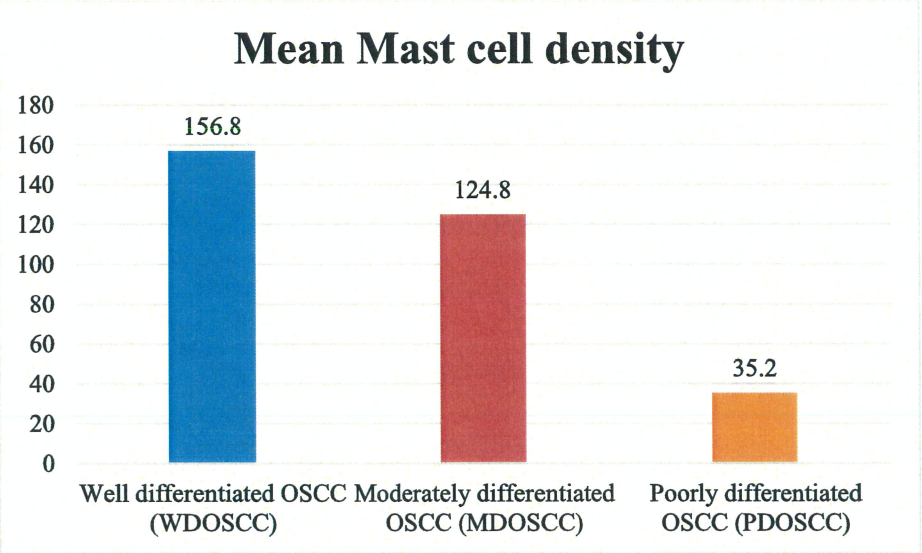
OBSERVATIONS AND RESULTS

OBSERVATION AND RESULTS

Table 3: Descriptives of Mast cell density with respect to different grades of OSCC

Mast cell density					
	N	Mean	Std. Deviation	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound
Well differentiated OSCC (WDSCC)	10	156.8000	6.74619	151.9741	161.6259
Moderately differentiated OSCC (MDSCC)	10	124.8000	6.74619	119.9741	129.6259
Poorly differentiated OSCC (PDSCC)	10	35.2000	6.74619	30.3741	40.0259

Table 3 showed the descriptives of Mast cell density with respect to different grades of OSCC. Poorly differentiated OSCC cases showed the minimum mast cell density while the WDSCC cases showed the maximum mast cell density.



Graph 1: mean mast cell density with respect to different grades of oral squamous cell carcinoma

Table 4: Intergroup comparison of mast cell density

ANOVA					
Mast cell density					
	Sum of Squares	df	Mean Square	F	P value
Between Groups	79462.400	2	39731.200	873.000	<0.001, S
Within Groups	1228.800	27	45.511		
Total	80691.200	29			

Intergroup comparison of mast cell density was done using one way ANOVA test. Overall, a statistically significant difference was observed. In other words, it can be said that the mean mast cell density was significantly different among all the three types of OSCC.

Table 5: Post hoc pairwise comparisons of mean mast cell density using Tukey's test

Multiple Comparisons						
Dependent Variable: mast cell density						
Tukey HSD						
(I) OSCC	(J) OSCC	Mean Difference (I-J)	Std. Error	P value	95% Confidence Interval	
					Lower Bound	Upper Bound
WDSCC	MDSCC	32.00	3.017	<0.001, S	24.5196	39.4804
	PDSCC	121.60	3.017	<0.001, S	114.1196	129.0804
MDSCC	WDSCC	-32.00	3.017	<0.001, S	-39.4804	-24.5196
	PDSCC	89.60	3.017	<0.001, S	82.1196	97.0804
PDSCC	WDSCC	-121.60	3.017	<0.001, S	-129.0804	-114.1196
	MDSCC	-89.60	3.017	<0.001, S	-97.0804	-82.1196

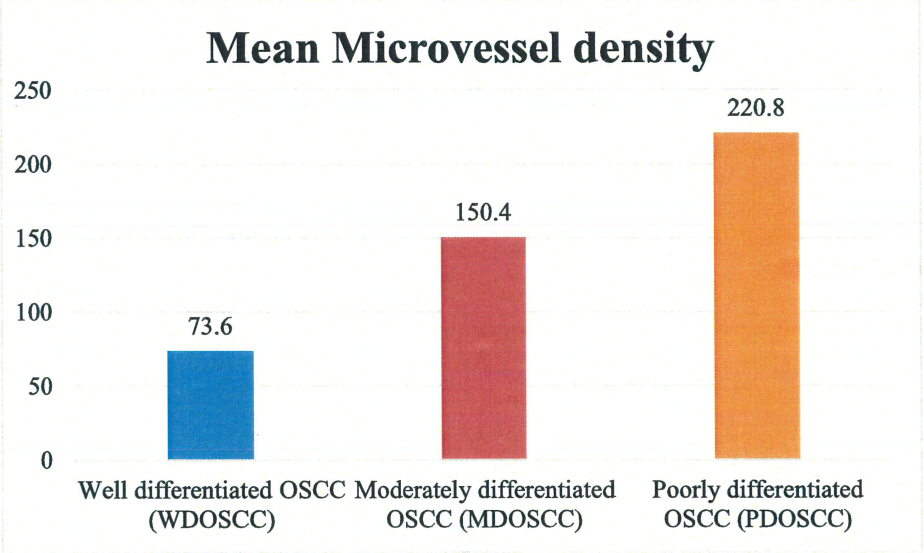
Post hoc pairwise comparisons of mean mast cell density using Tukey's test. It showed that the mean mast cell density of PDSCC cases was significantly lower than that of MDSCC, which was further significantly lower than that among WDSCC cases.

Table 6: Descriptives of Microvessel density with respect to different grades of OSCC.

Microvessel density					
	N	Mean	Std. Deviation	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound
Well differentiated OSCC (WDSCC)	10	73.6000	8.26236	67.6895	79.5105
Moderately differentiated	10	150.4000	8.26236	144.4895	156.3105

OSCC (MDSCC)					
Poorly differentiated OSCC (PDSCC)	10	220.8000	40.47715	191.8444	249.7556

Table 4 showed the descriptives of Microvessel density with respect to different grades of OSCC. WDSCC cases showed the minimum microvessel density while the PDSCC cases showed the maximum microvessel density.



Graph 2: Descripitives of Microvessel density with respect to different grades of oral squamous cell carcinoma

Table 7: Intergroup comparison of microvessel densit

ANOVA					
Micro vessel density					
	Sum of Squares	df	Mean Square	F	P value
Between Groups	108407.467	2	54203.733	91.615	<0.001, S
Within Groups	15974.400	27	591.644		
Total	124381.867	29			

Intergroup comparison of microvessel density was done using one way ANOVA test. Overall, a statistically significant difference was observed. In other words, it can be said that the mean microvessel density was significantly different among all the three types of OSCC.

Table 8: Post hoc pairwise comparisons of mean microvessel density

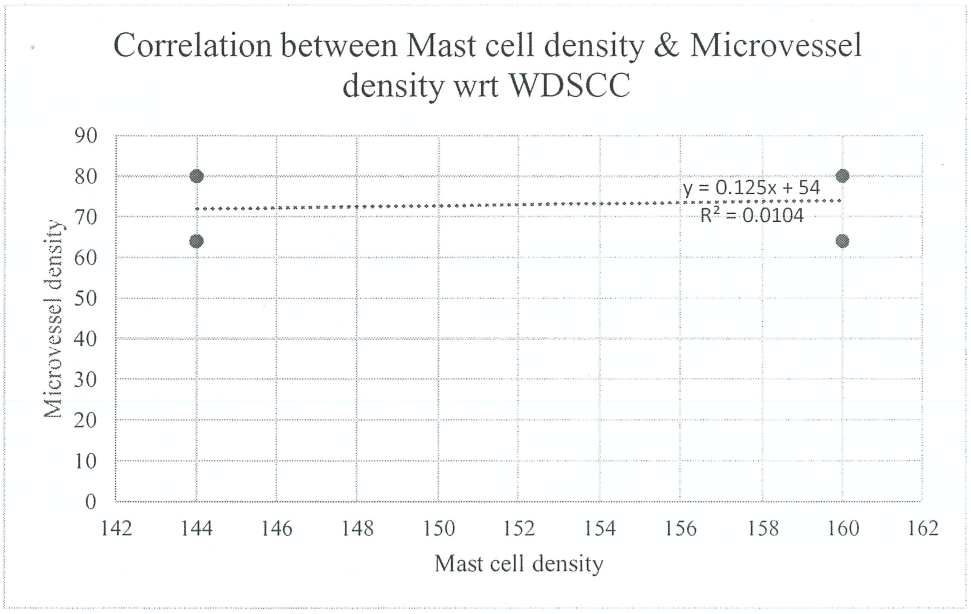
Multiple Comparisons						
Dependent Variable: Micro vessel density						
Tukey HSD						
(I) OSCC	(J) OSCC	Mean Difference (I-J)	Std. Error	P value	95% Confidence Interval	
					Lower Bound	Upper Bound
WDSCC	MDSCC	-76.80000*	10.87791	<0.001, S	-103.7709	-49.8291
	PDSCC	-147.20000*	10.87791	<0.001, S	-174.1709	-120.2291
MDSCC	WDSCC	76.80000*	10.87791	<0.001, S	49.8291	103.7709
	PDSCC	-70.40000*	10.87791	<0.001, S	-97.3709	-43.4291
PDSCC	WDSCC	147.20000*	10.87791	<0.001, S	120.2291	174.1709
	MDSCC	70.40000*	10.87791	<0.001, S	43.4291	97.3709

Post hoc pairwise comparisons of mean microvessel density using Tukey's test. It showed that the mean microvessel density of specimens with WDSCC was significantly lower than that of MDSCC, which was further significantly lower than that among PDSCC specimens.

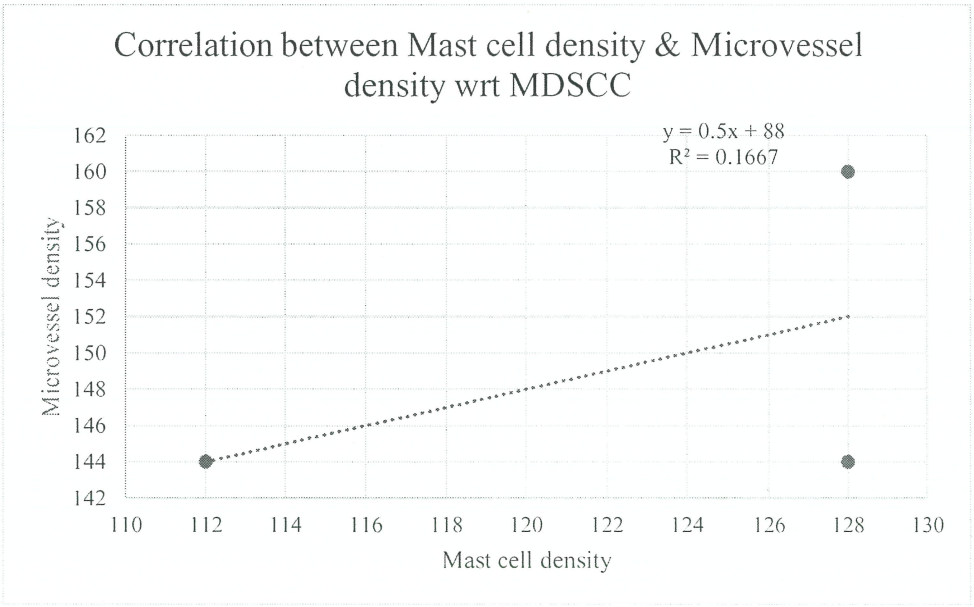
Table 9: Post hoc pairwise comparisons of mean microvessel density

	Correlation coefficient	P value
WDSCC	0.102	0.779, NS
MDSCC	0.408	0.242, NS
PDSCC	-0.375	0.286, NS
Overall	-0.894	<0.001, S

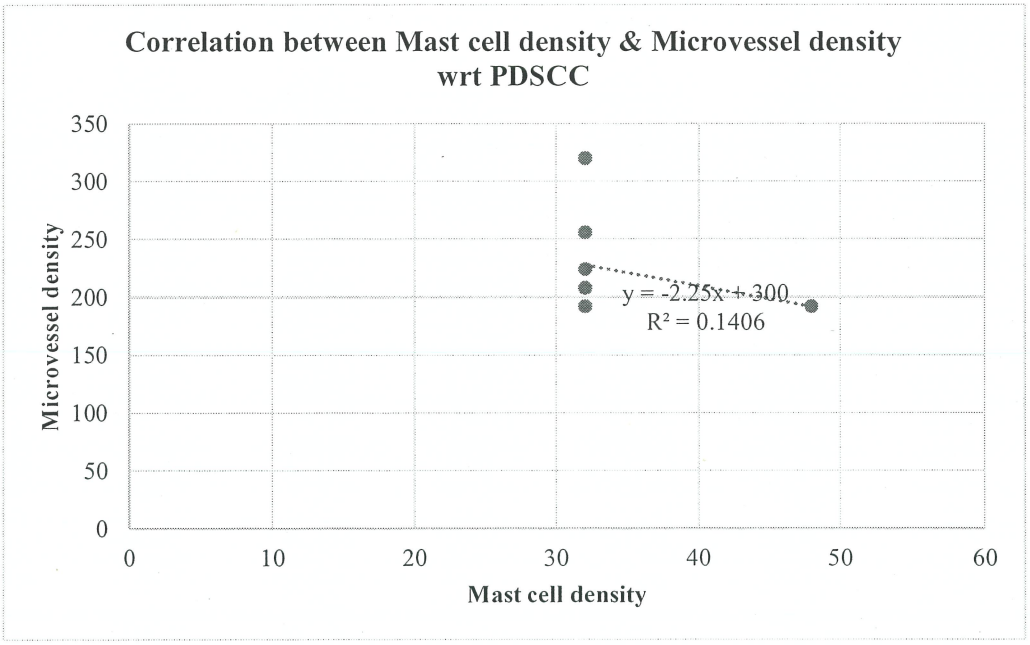
This table presents the correlation coefficients and corresponding p-values for the relationship between mast cell density & microvessel density among different grades of OSCC and overall. It showed that wrt WDSCC cases, a weak positive but statistically not significant, correlation was found between mast cell density & microvessel density. With respect to MDSCC cases, a moderate positive correlation was found which failed to reach the statistical significance. With respect to PDSCC cases, a moderate positive correlation was found, which was also not statistically significant. Overall, a strong negative correlation (statistically significant) was found between mast cell density & microvessel density.



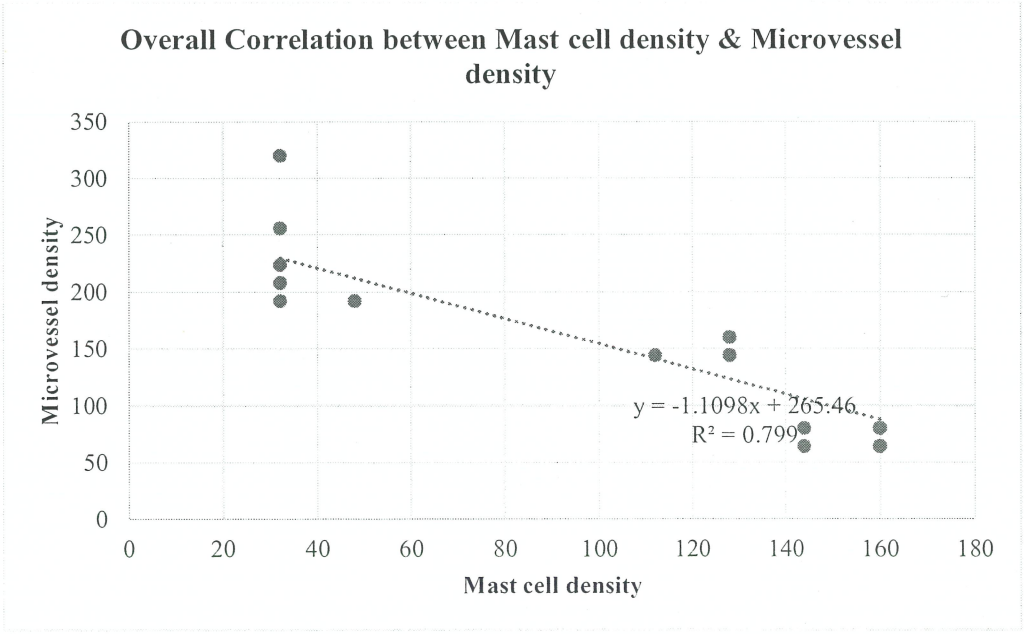
Graph 3: Scatter plot showing correlation between mast cell density & Microvessel density with respect to WDSCC



Graph 4: Scatter plot showing correlation between Mast cell density & Microvessel density with respect to MDSCC



Graph 5: Scatter plot showing correlation between Mast cell density & Microvessel density with respect to PDSCC



Graph 6: Scatter plot showing overall correlation between mast cell density & Microvessel density

DISCUSSION

DISCUSSION

Oral squamous cell carcinoma accounts for more than 90% of oral cancers with a poor 5year survival rate. Though OSCC is an epithelial malignancy, the invading tumour components are believed to be influenced by the tumour microenvironment which consists of tumour cells, immune cells like mast cells, macrophages, endothelial cells etc[62]. OSCC progresses by the breakdown of the basement membrane and extracellular matrix which facilitates local invasion, angiogenesis, vascular and lymphatic dissemination.

Angiogenesis is a phenomenon associated with physiological (embryogenesis, wound healing) and pathological events(neoplasia). It is determined by a balance between pro angiogenic (TGF B, FGF, TNF) and anti angiogenic(Angiostatin, Platelet factor) factors[62]. Pro angiogenic factors are secreted by host immune cells (mast cells) and tumour cells. Angiogenesis which is directly proportional to the microvascular density(MVD) can be assessed by marking the proliferating endothelial cells immunohistochemically with various endothelial markers like CD31, CD34, VEGF-A, Endoglin etc. Several markers have been used in the past; but we chose Endoglin(CD105), a cluster differentiation factor in the present study as it specifically stained the activated endothelial cells as compared to CD34 that stained all endothelial cells[77,82].

Endoglin is a powerful vasculogenic factor secreted by activated endothelial cells during formation of new capillaries making it the most specific diagnostic marker depicting angiogenesis in neoplasms. Upregulated CD105 is associated with areas of granulation tissue, tumor metastasis and shorter survival rates. Endothelial cells of tumour vessels show a significantly high rate of division when compared to normal

vascular channels which can be used to predict tumor behavior. Endoglin is a trans-membrane glycoprotein which is also a receptor for TGF beta signalling, induces endothelial cell proliferation, migration and fibrogenesis.[83]. Quantification of micro vessel density with Anti CD105 antibodies can aid in cancer treatment by controlling new blood vessel formation. Hence, it's use as a diagnostic marker and therapeutic target in cancer has to be explored.

We found micro vessel density (MVD) increasing proportionately with higher histological grades in OSCC. One way ANOVA test followed by Tukeys post hoc revealed that MVD was significantly higher in PDSCC compared to MDSCC & WDSCC. Our results were in accordance to the studies by Alivelu D & Basnekar M [84,85]. Tumour angiogenesis is driven not only by host derived signals, but also by factors secreted by tumour cells themselves which directly attract the endothelial cells to sprout towards the tumour[86]. **Mohtasham Nooshin et al**[87] have reported that angiogenesis is an un favourable survival indicator in oral cancers. **Nakandala K et al**[88] found elevated MVD in OSCC patients with increased nodal metastasis. A single luminal endothelial cell of a blood vessel will support more than 100 tumour cells during carcinogenesis[89] Sometimes, rate of tumour growth exceeds that of angiogenesis; hence in those cases, lesser MVD may be seen as tumour grows in size[62].

We believe that increased MVD in PDSCC could be due to tumour invasion in the stroma in the form of isolated cells or tiny groups, leading to multiple foci for initiation of angiogenesis.

Yet another indicator of angiogenesis is the presence of mast cells. In vitro studies have shown mast cells collect at sites of vascular tube formation[90]. MCD can

modulate micro vessel density in malignant tumours[82]. Mast cells originate in the bone marrow, circulate in peripheral blood and releases inflammatory mediators by degranulation. Activated mast cells play a role in mitosis, degradation of stroma, angiogenesis, upregulation of microvascular permeability and recruitment of phagocytic cells[91].**Telagi N et al**[92] in their study, grouped mast cells into typical (clear visualisation of nucleus, cell membrane and granules), atypical (clear visualisation of nucleus and granules without clarity of the cytoplasm) and granular (aggregations of granules without presence of nucleus). They reported a proportionate increase of degranulated mast cells in OSMF, OSCC and Oral leukoplakia with increase in inflammatory infiltration. Granulating mast cells were more than atypical and typical types in OSCC.

Activated Mast Cells secrete mediators that induce angiogenesis (VEGF), tumour proliferation, degradation of ECM etc. ECM degradation is aided by chymases and tryptases secreted by Mast Cells which provide space for the budding capillaries[93]. Mast cell growth factor which induces mast cell migration is synthesised by endothelial cell and epidermal keratinocytes[94,95]. Hence we believe, the increase in number of endothelial cells will also correlate with the mast cell numbers. Mast cell density (MCD) could be assessed by identifying the mast cells using toluidine blue, an easily available metachromatic dye.

We found MCD decreasing proportionately with increase in severity of OSCC which was in accordance to previously reported literature[21,59,96,97].

In our findings, MCD showed an inverse relationship with tumour grade, decreasing with advancing OSCC stages which was statistically significant.

This picture may be the result of failure of migration of mast cells in PDSCC due to reduced expression of chemotaxins or due to apoptosis of these cells in the tumour microenvironment. Reduced MCD in OSCC's when compared to epithelial dysplasia show an altered tumour microenvironment; their higher numbers in WDSCC shows a protective role of mast cells [98]. In addition, mast cells may be suppressed with the onset of malignancy or show reduced infiltration within tumour sites[62]. Anti tumorogenic role of Mast cells were reported in carcinomas of breast, prostate, stomach and lungs[99]. **Dantas RCM et al**[98] reported higher MCD in OSCC's that showed prolonged overall survival and improved prognosis. They also found higher MCD in WDSCC and MDSCC[99]. **Parizi AC et al**[100] reported that MCD is independent to degree of tumour differentiation in OSCC. **Das B, Mendonca SM**[101] in their study in adenocarcinoma of the prostate opined that mast cells can infiltrate solid tumours; and can be seen intra-tumourally and perilesionally around blood vessels. Based on their location, mast cells can be protumorogenic or antitumorogenic. They also found that the decrease in mast cells in high grade tumours could be attributed to the fact that these cells degranulate as the tumour proliferates. Mast cells can be tumorogenic or tumour suppressive. The latter features occur due to specific release of mediators in different diseases[100].

Oliviera-Neto HH et al[102] reported that establishment of a tumour environment would lead to failure of mast cell migration owing to down regulation of the c-kit activation pathway. Therefore we believe that evolution of the inflamed stroma(in dysplasia) to a tumour stroma (tumour microenvironment) could lead to reduced chemotaxins and increased apoptosis which reduced the MCD in poorly differentiated oral squamous cell carcinomas.

Nakandala K et al[88] reported a significant correlation between MCD and tumour site; where they found elevated MCD in tumours of buccal mucosa than the tongue. However, tumour site was not factored in the present study. They also opined that MCD may also be altered with different etiological factors that cause the tumour; for eg tobacco, trauma etc my have varying effects on mast cell levels..

Mast cell growth factor can be secreted by keratinocytes ; however, with the initiation of neoplasia, perhaps, the reduction in secretion of mast cell growth factor may ensue; owing to the reduced differentiation of the dysplastic epithelial tumour cells. This also could be the reason for reduced MCD with increase in grades of OSCC. Mast cells (with tryptase) seen within the tumour, may stimulate angiogenesis, while mast cells (with chymase) seen around the tumor may promote breakdown of the adjacent matrix and expand the invasion front[103].

A study in pulmonary adenocarcinomas reported that the initial role of mast cells could be to suppress neoplastic proliferation, which gradually shifts to promoting new blood vessel formation[104].

In the present study, we recorded a proportionate increase of MVD and a reduction in MCD with higher grades of OSCC. In general, a strongly negative correlation (statistically significant) was found between MCD & MVD in varying grades of OSCC. This could be attributed to the fact that OSCC is a multifactorial disease; and angiogenesis which is just one of the many factors promoting the tumour may be stimulated by numerous factors other than mast cells. Hence the relationship of MCD to MVD can be considered to be complicated. Few studies have shown a positive correlation between MCD and MVD[81,88]. However Kalra et al found no statistical significance in the correlation between these two parameters in OSCC[21].

A positive relationship between MVD and worse prognosis were reported by Oliveira et al[102] in tumors of head &neck. Targeting angiogenesis as an adjuvant therapy is supported by the fact that solid tumors need an efficient vascular supply to grow beyond 2 mm in size[105]. Also, supportive treatment methods that block mast cell degranulation may aid in limiting the progression of OSCC to higher grades. However, the role of mast cells remains controversial. Hence, we conclude that assessment of MVD and MCD can provide an insight in the use of anti-angiogenic agents in treatment strategies and predict the treatment outcome and survival of patients.

CONCLUSION

CONCLUSION

The present study was conducted using 30 samples of previously diagnosed OSCC which were retrieved from the archives. Three sections of each tissue were taken and stained with H & E, Toluidine Blue and CD105 (Endoglin).

10 samples of pyogenic granuloma were used as control. Microvascular density was assessed using Endoglin and Mast cell density was assessed using toluidine blue.

We found statistictically significantly increase in micro vessel density with increasing grades of OSCC and reduced Mast cell density with increase in grades.

Hence, we conclude use of antiangiogenic agents and drugs that can block Mast cell degranulation may aid in limiting the progression of Oral Squamous Cell carcinoma.

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APPENDICES

APPENDICES

ANNEXURE-I

BABU BANARASI DAS COLLEGE OF DENTAL SCIENCES

(Faculty of BBD University, Lucknow)

INSTITUTIONAL RESEARCH COMMITTEE APPROVAL

The project titled **"Expression of Endoglin in different grades of Oral squamous cell carcinoma and its correlation with mast cells: A retrospective comparative study."** submitted by **Dr. ANURADHA OJHA** Postgraduate student in the **Department of Oral Pathology &Microbiology** for the Thesis Dissertation as part of MDS Curriculum for the academic year 2022-2025 with the accompanying proforma was reviewed by the Institutional Research Committee in its meeting held on **16th October, 2023** 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 (IRC)
Principal
BBD College of Dental Sciences
BBD University, Lucknow

Lakshmi Bala 15/01/24

(Dr. Lakshmi Bala)
Co-Chairperson (IRC)
HOD Biochemistry
BBD College of Dental Sciences
BBD University, Lucknow.

ANNEXURE-II

BABU BANARASI DAS UNIVERSITY

BBD City, Faizabad Road, Lucknow – 226028 (INDIA)

Dr. Lakshmi Bala
Professor and Head, Deptt of Biochemistry and
Member-Secretary, Institutional Ethics Committee (IEC) of BBD University, Lucknow

Communication of the Decision of the VIIIth Institutional Ethics Committee Meeting

IEC Code: 15 **BBDU/MDS/15/2024**

Date: 07/03/2024

Title of the Project: Expression of Endoglin in different grades of Oral squamous cell carcinoma and its correlation with mast cells: A retrospective comparative study.

Principal Investigator: Dr. ANURADHA OJHA **Department: Department of Oral Pathology & Microbiology**

Name and Address of the Institution: BBD University, Lucknow
Type of Submission: New, MDS Dissertation proposal

Dear Dr. Anuradha Ojha
The meeting of the Institutional Ethics Committee (IEC) was held on 06-02-2024 in Conference room, First Floor, BBDCODS, BBD University, Lucknow. Following members were present:

1	Dr. Chandishwar Nath Rtd. Chief Scientist, CDRI, Lucknow.	Chairman
2	Dr. JS Srivastava, Rtd. Chief Scientist, CDRI, Lucknow.	Member
3	Dr. Manodeep Sen, Professor, Department of Microbiology, RMLIMS, Lucknow	Member
4	Dr. Shaleen Chandra, Professor & Dean, Dental Sciences, Atal Bihari Vajpai Medical University (ABVM University), Lucknow	Member
5	Dr. Manuka Khanna, Professor, Deptt. of Political Science, Lucknow University, Lucknow	Member
6	Mr Abhishek Chaudhary, Advocate, Lucknow	Member
7	Dr. Puneet Ahuja, Professor of Oral Pathology and Principal, BBD College of Dental Sciences, BBD University, Lucknow	Member
8	Dr. Lakshmi Bala, Professor & Head, Department of Biochemistry, BBD College of Dental Sciences, BBD University, Lucknow	Member-Secretary

The committee reviewed and discussed your submitted documents of the research proposal in the meeting.
Decisions: The committee provided waiver to obtain consent for the above proposal.

Lakshmi Bala 07/03/24

Dr. LAKSHMI BALA
Member-Secretary
Institutional Ethics Committee
BBD University, Lucknow-28

(Dr. Lakshmi Bala)
Member-Secretary IEC
BBD University
Ethics Cell (Vth Floor, BBDCODS)
ethics@bbdu.ac.in

ANNEXURE-III

OBSERVATIONS

Cases	Toludine Blue – No of Mast Cells	Toludine Blue – No of Mast Cells	CD 105-No of Positive Blood Vessels	CD 105-No of Positive Blood Vessels
	Observer 1	Observer 2	Observer 1	Observer 2
WDSCC 01	10, 10, 10, 8, 8	10, 8, 10, 8, 8	7, 2,4,4, 5	4,4,4,4,5
WDSCC 02	10, 8, 10, 7, 10	10, 8, 8, 7, 10	8, 5, 4, 5, 4	5, 5, 4, 5, 4
WDSCC 03	10, 10, 10, 10, 7	10, 10, 10, 7, 10	5,5, 5,8,5	5,5, 5,8,5
WDSCC 04	9, 10, 9, 10, 10	10, 8, 10, 10, 7	5,5,5,5,5	4,4,5,5,5
WDSCC 05	10, 10, 10, 9, 10	9, 10, 10, 10, 10	5,4,4,4,5	4,4,4,4,5
WDSCC 06	9, 8, 10, 10, 7	8, 10, 10, 9, 10	4,4,4,4,5	8,5, 5,8,5
WDSCC 07	10, 10, 9, 10, 10	9, 10, 10, 8, 8	5, 5, 4, 5, 4	8,5,5,5,5
WDSCC 08	8, 10, 10, 9, 10	10, 9, 10, 7, 10	8,5, 5,8,5	4,4,4,4,5
WDSCC 09	8, 10, 10, 8, 10	10, 10, 10, 10, 10	4,5,5,5,5	5,5,5,4,5
WDSCC 10	10, 10, 10, 7, 10	10, 10, 9, 10, 10	5,4,4,4,4	5,5,5,5,5
MDSCC 01	8, 8, 7, 7, 8	8,7,6,8,8	8, 9,10, 10, 10	10,9,9,7,9
MDSCC 02	8, 8, 8, 8, 7	7, 8, 8, 8, 7	9, 10, 10,9,9	10,10,10,7,9
MDSCC 03	8, 8, 8, 8, 6	7, 8, 8, 8, 6	10, 2, 9, 3, 9	7, 10, 10,9,9
MDSCC 04	8,8,8,8,8	8,8,8,8,8	10, 9, 10,10,10	9, 2, 9, 3, 9
MDSCC 05	8,7,6,8,8	6,7,6,8,8	10,9,9,7,9	9, 9, 10,10,10
MDSCC 06	7, 7, 8, 8, 7	6, 8, 8, 8, 6	9, 10, 10,9,9	10,9,9,7,10
MDSCC 07	7, 8, 8, 8, 6	9,8,8,8,8	9, 2, 9, 3, 9	10, 2, 9, 3, 10
MDSCC 08	6, 8, 8, 8, 7	8,8,6,8,8	9, 9, 10,9,9	9, 9, 10,10,10
MDSCC 09	7, 7, 7, 8, 6	9, 8, 8, 8, 8	10, 2, 9, 9, 9	10,9,9,7,9

MDSCC 10	7,7,8,8,8	8, 8, 8, 8, 6	9, 9, 10,10,10	9, 2, 9, 10, 10
PDSCC 01	3, 2, 3, 2, 2	2, 2, 2, 2, 3	12, 14, 14, 12, 13	13, 13, 13, 12, 12
PDSCC 02	3, 2, 2, 2, 3	3,3,3,3,3	16, 12, 14, 14, 12	15,13,12,13,13
PDSCC 03	3, 2, 2, 2, 3	3, 3, 2, 2, 3	12,12,12,13,13	13, 13, 12, 12, 12
PDSCC 04	3,3,3,3,2	3, 3, 2, 2, 3	12, 13, 12, 12, 12	13,13,13,13,13
PDSCC 05	3, 3, 2, 2, 2	2,3,3,3,2	13,12,20,20,20	13, 13, 12, 12, 12
PDSCC 06	3, 2, 2, 2, 3	2, 2, 2, 2, 3	15, 12, 14, 14, 15	13, 16, 14, 14, 12
PDSCC 07	2,3,3,3,2	2,3,3,2,2	15,12,12,13,15	16,12,12,13,13
PDSCC 08	2, 2, 2, 2, 2	3, 3, 2, 2, 2	13, 13, 12, 12, 12	15, 13, 12, 12, 12
PDSCC 09	2,2,2,3,2	2,3,3,3,2	13,12,20,20,20	12,12,20,20,20
PDSCC 10	2, 2, 3, 2, 3	2, 3, 3, 3, 3	15, 15, 15, 12, 12	15,13,12,13,20

ANNEXURE-IV

STATISTICAL 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. All the variables were continuous variables, which were summarized as mean and standard deviation. Graphs were prepared on Microsoft Excel.

Normality of the data was checked by **Shapiro Wilk test**. Data was found to be normal. Keeping in view the nature (continuous) & distribution (normal) of data, inferential statistics were performed using **parametric tests of significance**.

Inferential statistics were performed using **One way Analysis of Variance.** Post hoc pairwise comparison was done using **Post hoc Tukey'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

$\sum X$ = Sum of all individual observations

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

n = Total number of observations

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 numbers which act 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

Pearson correlation coefficient

Pearson Correlation coefficient is used to find how strong a relationship is between data. It is a statistic that measures linear correlation between two variables. The formulas return a value between -1 and 1, where:

- 1 indicates a strong positive relationship.
- -1 indicates a strong negative relationship.
- A result of zero indicates no relationship at all.

Pearson's correlation coefficient is the covariance of the two variables divided by the product of their standard deviations. The form of the definition involves a "product moment", that is, the mean (the first moment about the origin) of the product of the mean-adjusted random variables.

$$r_{xy} = \frac{\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2}\sqrt{\sum_{i=1}^{n}(y_i - \bar{y})^2}} \quad \text{(Eq.3)}$$

ANNEXURE-V

PLAGIARISM REPORT

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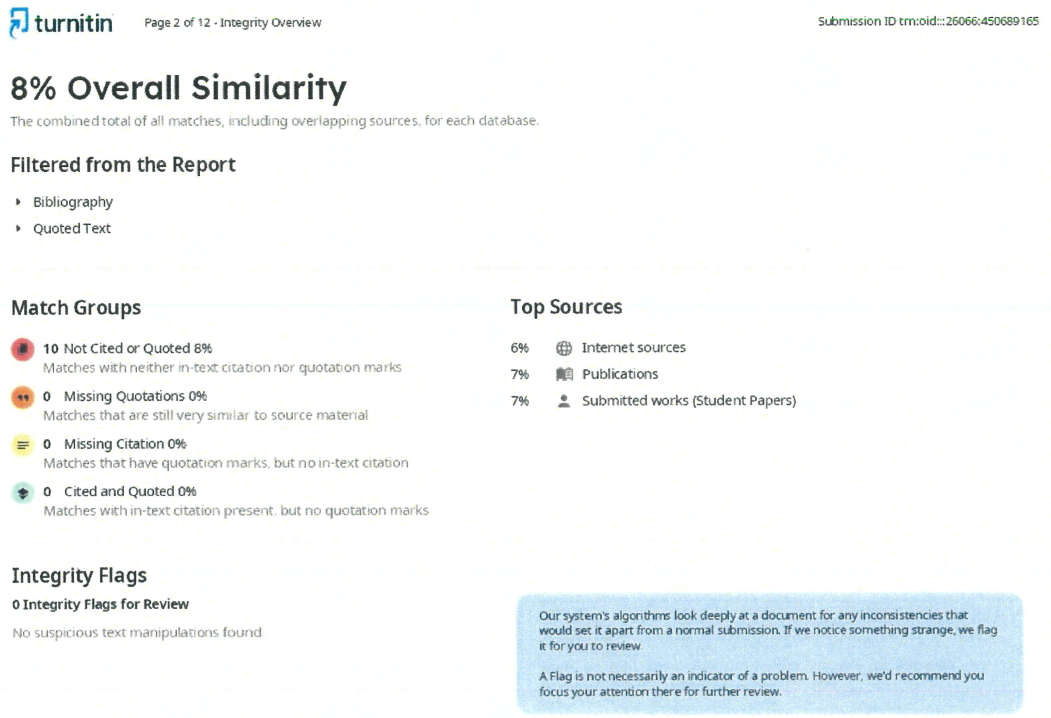
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