RESEARCH PAPER

HISTOPATHOLOGICAL COMPARATIVE ANALYSIS OF MAST CELLS IN NORMAL MUCOSA, ORAL POTENTIALLY MALIGNANT DISORDERS AND ORAL SQUAMOUS CELL CARCINOMA WITH SPECIAL STAINS

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ABSTRACT

Prolonged inflammation is a primary factor implicated in the pathogenesis of many oral pathologic entities, such as oral epithelial dysplasia, oral lichen planus, and oral squamous cell carcinoma (OSCC). Mast cells are immune cells with a significant role in the disease process of many inflammatory diseases. They have been identified in Oral potentially malignant disorders (OPMDs) and OSCC, but their role in these conditions remains unclear. The aim of the study is to determine the significance of mast cells by quantifying them in normal and altered oral tissue, and to assess the staining effectiveness of Toluidine blue and Methylene blue for mast cell visualisation. The present retrospective study was conducted in the Department of Oral Pathology, Sree Mookambika Institute of Dental Sciences, Kulasekharam, Kanyakumari district, from January 2022 to April 2024. Archival, histologically confirmed cases, including eleven normal mucosa, eleven OPMDs, and eleven OSCC specimens, were collected and analysed for mast cell quantification using Toluidine blue and Methylene blue staining. The staining potential of the two stains was compared within each study group based on mast cell counts obtained. Statistical analysis was done using one-way ANOVA to assess the significance of the results. Of the 33 samples included in the study, 58% of the samples were obtained from male patients, and the remaining 42% samples were obtained from female patients. OSCC had the lowest mean mast cell count, while OPMDs had the highest mean count. The mean mast cell counts for normal mucosa, OPMDs and OSCC using methylene blue and toluidine blue stains were 1.27±0.64 and 1.01±0.60; 3.87±2.75 and 3.14±1.74 and 3.18±1.91 and 3.15±1.23, respectively. Statistical analysis revealed a significant difference in all groups with a p-value of less than 0.05. Methylene blue stained the mast cells more intensely than toluidine blue in all three groups of tissue specimens. The rise in mast cell counts from normal tissue to OPMDs and their progressive decline from OPMDs to OSCC highlights their potential protective role. Accurate quantification of mast cells using special stains may provide valuable prognostic insights into the evolution of disease from oral potentially malignant disorders to oral cancer.

Keywords: mast cells, Methylene blue, Oral epithelial dysplasia, Oral lichen planus, Oral potentially malignant disorders, Oral squamous cell carcinoma, Toluidine blue

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INTRODUCTION

Head and Neck squamous cell carcinoma (HNSCC) is ranked as the sixth most prevalent cancer worldwide, and among the reported cases of HNSCC, Oral squamous cell carcinoma (OSCC) is the most prevalent subtype, with a yearly incidence of more than 350,000 cases. A recent projection from the Global Cancer Observatory (GCO) reported that by 2040, the incidence of OSCC will amplify by almost 40% with a corresponding rise in the mortality rate.[1] There are multiple risk factors involved in the causation of OSCC included among which are Oral potentially malignant disorders(OPMDs). The WHO defined them in 2005 as any abnormality in the oral cavity associated with a higher risk of developing into oral cancer, and some notable OPMDs include oral lichen planus (OLP), erythroplakia, leukoplakia, and oral submucous fibrosis (OSMF).[2]

Inflammation is a double-edged sword. Even though it is the host defence mechanism against external pathogens, chronic inflammation can be detrimental to normal cellular function and

can help promote pathology. Even in the case of OPMDs and OSCC, the pathogenesis of these conditions is initiated by inflammation regulated by cells involved in the immune response, including mast cells, lymphocytes, neutrophils and macrophages.[3] A review article in 2021 stated that oral inflammatory conditions like OLP and OED contain a proinflammatory milieu of cytokines, chemokines and other molecules in their microenvironment produced by various immune cells. Each of these components has a specific function that helps in disease progression. Therefore, understanding their role could be beneficial in using these inflammatory cells as prognostic biomarkers or in formulating targeted therapy against OSCC.[4]

Mast cells have a myeloid origin. They are granulated cells that have diverse functions that are influenced by their microenvironment.[5] Identified by Paul Ehrlich in 1878, they are implicated in several allergic disorders and may also contribute to other pathological conditions such as immunodeficiencies, microbial infections, and even certain types of cancer.[6] Tumour-associated mast cells can have either pro or anti-tumorigenic effects. Studies have found that increased mast cell density in a solid tumour microenvironment (TME) can be indicative of a positive prognosis in a few cancers, namely ovarian cancer and diffuse B-cell lymphoma, while present with ambivalent prognostic value in other types of cancers, namely melanoma, lung and breast cancer.[7] But in the case of oral cancer, the prognostic significance of mast cells is poorly understood.

Mast cells are best visualised with metachromatic dyes that absorb light at different wavelengths depending on their environment and concentration without changing their chemical structure. Routine hematoxylin and eosin staining of mast cells is limited by the presence of heparin and histamine in them. The heparin in the granules is responsible for the metachromatic property of the mast cells. The most prevalent dye used for mast cell staining is Toluidine blue. It is characterised as a hydrophilic weak cationic dye that binds to nucleic acids and glycosaminoglycans (GAG) to produce a purple stain in mast cells when applied as mild acidic aqueous solutions.[8] A less common cationic dye alternative that also stains nucleic acids is Methylene blue. As a phenothiazine derivative, it renders a blue stain to mast cells when used as an aqueous solution.[9] Both these dyes have been tested in supravital staining of oral tissues to help in the early identification of OPMDs and malignancy. The utilization of toluidine blue staining as a supplementary tool for early diagnosis of OSCC due to its low cost and accessibility has been recommended by many studies. But methylene blue, which is less toxic than toluidine blue has limited studies to assess its application for early detection of oral lesions.[10] The present study addresses this gap and aims to assess the diagnostic potential of methylene blue as a staining agent to study mastocytes in different oral conditions in comparison to Toluidine blue.

Early detection of OPMDs and OSCC can greatly help the prognosis of the disease. But the influence of mast cells in tumorigenesis and the validity of staining methods are sparsely explored. The current study aims to correlate mass cell density with disease progression spanning from normal mucosa, and OPMDs to OSCC, as well as to evaluate the diagnostic reliability of methylene blue as an alternative mast cell staining solution. The central research question was whether mast cell counts vary significantly across tissue samples in different disease conditions and if Methylene blue has a staining efficacy comparable to or different from Toluidine blue. The null hypothesis stated that mast cells do not influence tumorigenesis in oral tissues, and methylene blue does not differ significantly from toluidine blue in its ability to stain mast cells in oral tissues. The alternate hypothesis is that mast cells impact the tumorigenesis in oral tissues, and methylene blue differs in its staining efficacy from Toluidine blue.

MATERIALS AND METHODS

The present retrospective comparative histopathological short study was conducted in the Department of Oral Pathology, Sree Mookambika Institute of Dental Sciences, Kulasekharam, Kanyakumari district, from January 2022 to April 2024 after obtaining ethical approval from the Institutional Ethics Committee (Ref. No.44/2024).

SAMPLE SIZE CALCULATION

A minimum sample size of 33 specimens, with 11 specimens in each group, was calculated using G*Power software version 3.1.9.7 (Heinrich Heine University, Düsseldorf, Germany). The calculation was based on a one-way ANOVA model, assuming an effect size of 0.805 derived from a previous study,[3] a statistical power of 80%, and a significance level (α) of 0.05 for a two-tailed test. A convenient sampling method was employed.

INCLUSION AND EXCLUSION CRITERIA

Paraffin-embedded blocks of histopathologically proven cases of Normal oral mucosa, OPMDs, namely different grades of oral epithelial dysplasia (OED) and oral lichen planus (OLP) and OSCC were included in the study. Histopathological slides with areas of tissue folding/artefacts and samples from extra-oral sites were excluded from the study.

STUDY PROCEDURE

Paraffin-embedded blocks from all study groups were cut into 5 µm-thick tissue sections using a microtome and mounted on adhesive-coated glass slides. A total of 66 tissue sections were prepared for the study.

Slide preparation of all sections began by placing the slides on a slide warmer to ensure proper adhesion of the specimen to the slide by melting the wax. Deparaffinization was done by placing the slides in a Coplin jar containing xylene (Himedia Laboratories, Thane, India). Three changes of xylene, each for 5 minutes, were done. Rehydration of the sections was done by dipping each slide 10 times in serial grades of alcohol (100%, 90%, 70%, 50%). This was followed by rinsing each slide in rinsing water.

For toluidine blue staining, 33 tissue sections, including all three study groups, were immersed in the toluidine blue stain (0.1-1%) (Himedia Laboratories, Thane, India) for 10 seconds, washed under running tap water, differentiated in 100% alcohol for 2 minutes, followed by clearing with xylene and mounted in DPX for microscopic evaluation.[11]

Since no specific staining protocol was available for methylene blue staining of oral tissues, the remaining 33 tissue sections were immersed in the methylene blue stain (0.1-1%) (Himedia Laboratories, Thane, India) for 1–2 minutes, washed under running water, differentiated in 100% alcohol for 10–15 seconds, cleared in xylene, and mounted in DPX to be visualised under the microscope.

The mast cells were visualised using a scientific microscope. Mast cells appeared as round-to-oval cells with a deep blue granular cytoplasm in methylene blue staining [Figure- 1:(A), 2:(A), 3:(A)], while they appeared as cells that are round-to-polygonal in shape with a central nucleus and a granular cytoplasm that stained purple with toluidine blue staining [Figure- 1:(B), 2:(B), 3:(B)].

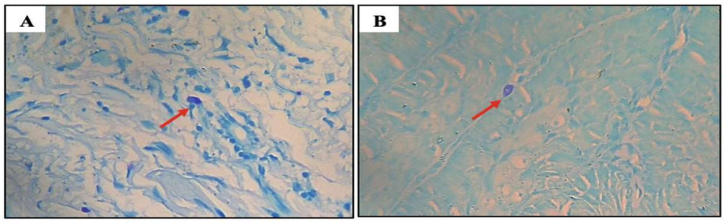

Figure 1: (A) Methylene blue and (B) toluidine blue-stained section showing a mast cell (Red arrow) in Normal mucosa (40x magnification)

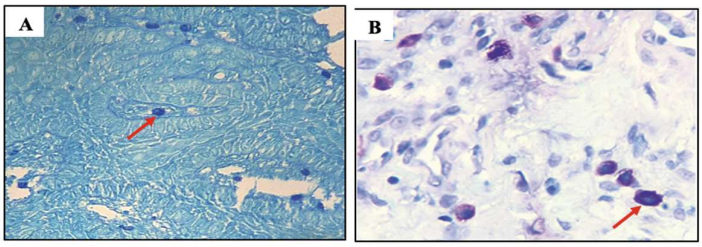

Figure 2: (A) Methylene blue and (B) toluidine blue-stained section showing mast cells (Red arrow) in OPMDs (40x magnification).

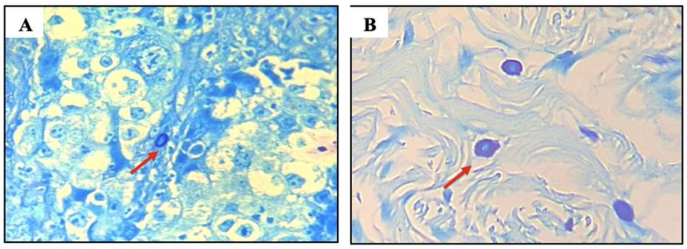

Figure 3: (A) Methylene blue and (B) toluidine blue-stained section showing mast cells (Red arrow) in OSCC (40x magnification)

The mast cell count in each study group was done by selecting random non-overlapping five fields, and photomicrographs of these fields were captured at high-power magnification using a research microscope. To reduce observational bias, the same images were assessed for mast cell visualization thrice by two investigators. The average of all mast cell quantifications was used for statistical assessment.

STATISTICAL ANALYSIS

All the collected samples were compared and analyzed using the ANOVA test. The software used was IBM SPSS Statistics Version 28. The results obtained from these tests were statistically significant if the p-value was <0.05.

RESULTS

The demographic assessment of the study samples revealed that samples in all study groups were predominantly male, and the average participant age ranged from 30 to 60 years.

The mean mast cell count assessment in the study groups using methylene blue and toluidine blue is summarised in [Table-1] and [Table- 2], respectively.

Table 1. Comparison of mean mast cell count in methylene blue.

Group	Methylene blue (Mean ± SD)	P value
Normal mucosa	1.27±0.64	0.012*
OPMDs	3.87±2.75	0.012*
OSCC	3.18±1.91	0.012*

The p-value obtained was 0.012. *p<0.05 – Statistically significant

Table 2. Comparison of mean mast cell count in toluidine blue.

Group	Toluidine blue (Mean ± SD)	P value
Normal mucosa	1.01±0.60	<0.001† **
OPMDs	3.14±1.74	<0.001† **
OSCC	3.15 ±1.23	<0.001† **

On assessment, the p-value obtained was 0.001.† p<0.005-Highly significant

The findings revealed that the mean mast cell count was lowest in the control— normal mucosa samples when compared to disease groups, OPMDs, and OSCC samples. Within the disease groups, samples from OPMDs had a higher mast cell count than OSCC samples. Statistical evaluation of these values resulted in a p-value of 0.012 in the case of methylene blue and 0.001 in toluidine blue, respectively, which were statistically significant (p-value <0.05).

In addition to this, the mean mast cell counts values obtained were also used to assess the staining capacity of methylene blue and toluidine blue to stain mast cells within each group, as shown in [Table 3].

Table 3. Comparison between two stains.

	Groups	N	Mean	Std. Deviation	Std. Error Mean	Mean difference	P value
Normal mucosa	Methylene blue	11	1.2727	.64667	.57680	-0.0727	0.917
Normal mucosa	Toluidine blue	11	1.0127	.60407	.37279	-0.0727	0.917
OPMDs	Methylene blue	11	3.8727	2.75430	.83045	0.7273	0.468
OPMDs	Toluidine blue	11	3.1455	1.74606	.52646	0.7273	0.468
OSCC	Methylene blue	11	3.1818	1.91302	.19498	-0.40	0.567
OSCC	Toluidine blue	11	3.1545	1.23642	.65852	-0.40	0.567

The comparison among two stains in normal mucosa resulted in a p-value of 0.917, in OPMDs resulted in a p-value of 0.468, and in OSCCs resulted in a p-value of 0.567. As p>0.05, none of them were statistically significant.

Based on mast cell counts alone, it was found that methylene blue had stained more mast cells in all study groups when compared to toluidine blue. But statistical tests within each group revealed it was not statistically significant, as the p-value was > 0.05 in all the groups. Therefore, in the present study, neither stain had a significant difference in the staining capacity of any cell assessed.

DISCUSSION

The incidence and prevalence of OSCC have been on the rise, with staggering projections for the next few years. Therefore, there is a need for the prevention of OSCC development and to curb its progression. The detection of precursor conditions, such as OPMDs and identifying OSCC in its nascent stages, can significantly improve prognosis and reduce the mortality rate, as discussed earlier.

Numerous biomarkers have been identified to aid in this process, but there is always a need for cheaper, accessible, simpler-to-perform alternative methods. The use of special stains such as toluidine blue and methylene blue is one such method. Even though many studies have assessed the feasibility of toluidine blue as a special stain, methylene blue staining has not been extensively evaluated for its potential.[3,9-12]

The role of chronic inflammation as a potential trigger for cancer initiation and progression was first postulated by Rudolf Virchow in the year 1863. Since then, numerous studies have been done to understand this relationship. If the role of immune cells in this partnership can be better understood, it can open avenues for numerous diagnostic, therapeutic, and prognostic strategies.[12,13]

Mast cells are an important component of the immune response. They are usually triggered in response to an allergic reaction, but their presence in the circumvascular regions and their interaction with other cells have been found to impact numerous aspects of tumourigenesis. These include mitotic activity, tissue remodelling, angiogenesis, and immunomodulation.[12,13] However, their role in tumourigenesis is dependent on tumour type, their location within the tumour microenvironment (TME), stage of mast cell activation, and the interplay between pro and anti-tumourigenic actions on tumour cells.[7]

The present study evaluated the mast cell count in OPMDs (oral epithelial dysplasia & lichen planus) and OSCC in comparison with normal oral mucosa using histological staining with methylene blue and toluidine blue, and further assessed the staining capacity of both dyes based on the available data.

The assessment of demographic details of age and gender in the present study revealed that most of the study participants were males across all groups, with an average age of 30 years, 54.1 years and 60 years in normal oral mucosa (NOM), OPMDs and OSCC, respectively. These findings were corroborated by the studies conducted by Singh et al.[11] and Shreshta A. et al.[3] where the study participants were primarily males and presented with a similar age range across the same study groups.

The present study revealed that mast cell counts increased in disease states when compared to normal oral mucosa, with the highest counts seen in OPMDs, followed by OSCC with both toluidine blue and methylene blue.

This presentation of the maximum number of mast cells in OPMDs, when compared to OSCC and NOM, is attributed to the phenomenon known as “Piece meal degranulation” exhibited by mast cells. By this characteristic feature, in certain instances, mast cells gradually secrete specific mediators through selective cellular pathways instead of the rapid, complete release of their granular contents on stimulation.[7]

The increase in the number of mast cells observed in OED than in OSCC and normal mucosa is comparable to results from research by Singh et al.[11] and Atarbashi-Moghadam S. et al.[12] wherein the highest mast cell count was seen in OED, followed by OSCC, and the least mast cell count was seen in NOM. This phenomenon was explained by the authors as an effect of the mast cell activation and degranulation. The interleukin-1 released helps in cellular proliferation, while the histamine increases the permeability of the mucosa and improves access to the connective tissue for the irritants to result in epithelial dysplasia.

The increased mast cell counts in OLP compared to the NOM and OSCC corroborate the findings of Shreshta A. et al[3], who reported elevated levels of mast cells in OLP in comparison to NOM and OSCC. The author attributed it to mast cell degranulation initiated by the major histocompatibility class I-associated CD8 cell activation in OLP. This initiated the release of TNF-alpha, which further aids in the release of matrix metalloproteinase-9 (MMP-9) that damages the basement membrane and helps in the progression of OLP. Also, TNF-alpha

continues its pro-tumourigenic action by maintaining a continuous release of mast cells through the RANTES (regulated upon activation normal T cell expressed and secreted) pathway. The study by Ai et al.[14] suggested that the histamine released by the degranulation of mast cells increases the permeability of the surrounding vasculature, which results in oedema and an increase in T cells. These lymphocytes initiate the degeneration of basal cells and keratinocytes, which results in the formation of Civatte bodies seen in OLP.[15]

The elevated mast cell counts in OSCC when compared to NOM, but still lower than OPMDs in the current study, agreed with the results obtained by multiple studies in the past. The possible reason for this reduction in mast cell count in comparison to OPMDs is that there was a failure in the migration of mast cells once a TME was properly established in the carcinomatous lesion. The authors postulated that the chemotactic factors were reduced in the TME or there was a downregulation in the c-kit activation pathway that was necessary for mast cell recruitment.[3,11] However, few studies have presented results contradicting the findings of the present study. Studies by Iamaroon et al.[15] and Michailidao et al.[16] suggested that the mast cell count was higher in OSCC when compared to OPMDs. This increase was attributed to an angiogenic switch that was initiated by mast cells during the early stages of malignant transformation. These findings suggest that mast cells have a dual role in tumorigenesis.

Assessment of the staining capacity of toluidine blue and methylene blue revealed that methylene blue has stained more mast cells within all groups when compared to toluidine blue, but the lack of statistical significance ($p>0.05$) suggests that both stains have similar staining capacity and can be used based on their availability. The possible reason for reduced staining by toluidine blue can be attributed to the reduced heparin present in mucosal mast cells when compared to connective tissue mast cells.[17]

The strength of the current study lies in the dual focus on both biological and methodological aspects. Mast cell density was systematically quantified across all study groups, while staining efficacy of both stains was comparatively evaluated.

The present study has its limitations as well. The study did not include other confounding factors, such as grades of OPMDs and OSCC, that can influence the outcome of the study. The single-centre and retrospective design increase the risk of confounding variables and external validity. Mast cell quantification depended on manual methods, which may introduce observer bias. Future studies with larger, multicentric cohorts, improved with grade differentiation within the samples, and automated image analysis can aid in validating the findings of this study and exploring the potential role of mast cells as prognostic and diagnostic marker in Oral Pathology.

CONCLUSION

Within the limitations of the current retrospective study, the mast cell concentration was found to increase from normal mucosa to OPMDs and subsequently decrease in OSCC. These findings suggest that further studies into the contributory role of mast cells with bigger sample sizes can help in devising diagnostic and therapeutic strategies to combat OSCC. The staining capacity of both toluidine blue and methylene blue was comparable, indicating that both are equally effective in the visualisation of mast cells guided by practical consideration rather than performance.

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