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Paper Title: Evaluation of Mast Cells in the Lesions Involving Oral Cavity through Modified Ferroin Technique

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Abstract:
Background: *Mast cells are considered to be multifunctional cells of immunity presenting themselves in several health and disease states. It is increasingly evident that maturation, function, and phenotype of mast cells are a direct result of the local microenvironment. Degranulated mast cells that are in active form help in knowing the chronicity of lesion and thus aiding in the treatment planning for better diagnosis. The anti-mast cell therapy may offer an adjunct to the existing treatment modalities in the coming years. Modified Ferroin Technique, stain only the degranulated mast cells aiding in detection of chronicity of the lesion.*
Objective: *To stain mast cells using Modified Ferroin Technique and evaluate the activity of mast cells.*
Materials and Methods: *Total number of 30 Cases were divided into three groups with each 10,* ***Group 1****-Oral Reactive lesions,* ***Group 2****- Oral lichen Planus and* ***Group 3****- Oral SubmucousFibrosis. Two sections of 4 ⁄m thickness from formalin fixed paraffin embedded tissues were obtained and stained with Toludine Blue O and Modified Ferroin Technique.*
Results: *Mast cells exhibited intense orange color cytoplasm with bluish violet nuclei with Modified Ferroin Technique. 30 cases positively stained for Toludine Blue O . 6 cases positively stained for Modified Ferroin Technique including only Group 1 and negatively stained for Groups 2 and 3.*
Statistical Analysis: *Fischer Exact Test and Spearman Rank Correlation*
Conclusions: *Modified Ferroin Technique, stain degranulated mast cells that are in active form helping in knowing the chronicity of lesion and thus aiding in the treatment planning for better diagnosis.The anti-mast cell therapy may offer an adjunct to the existing treatment modalities in the coming years as these cells havepotential to not only act as primary responders in pathology but also to react to changes in the environment by communicating with other cells implicated in physiology and immunology including innate, adaptive immunity and immune tolerance.*
Keywords: *Immunity , Mast cells, Modified Ferroin.*
Conflict of Interest: *None*

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I. Introduction

Mast cells were first described by Paul Ehrlich in 1878. Mast cells have been analyzed as effectors of allergy, predominantly in acute phases of allergic reactions.[1] Early research on mast cells relied on morphological features to identify their distribution in physiological and pathological states. The functional implications of Ehrlich's original view of these cells were being metachromatic and granulated implicated in the nutrition of the adjacent tissue evolved gradually.In 1937, mast cells were described for enriched heparin content. The following years witnessed many research studies establishing relationship between mast cells, histamine, and anaphylaxis.[1] In 1967 , it was observed that IgE was capable of mediating the release of histamine from sensitized tissue mast cells.[1] Currently, mast cells are considered to be multifunctional cells of immunity presenting themselves in several health and disease states. It is increasingly evident that maturation, function, and phenotype of mast cells are a direct result of the local microenvironment. It is also observed that they have a marked influence on their ability to particularly recognize and react to various stimuli by releasing an array of biologically active mediators.[1] The widespread tissue distribution and adaptability of mast cells endow them with the potential to not only act as primary responders in pathology but also to react to changes in the environment by communicating with other cells implicated in physiology and immunology including innate, adaptive immunity and immune tolerance.[1]

Oral mucosa is subjected constantly to internal and external stimuli and hence manifests a spectrum of diseases that range from reactive, developmental, inflammatory to carcinogenic.[2]The mast cell count is raised

in oral inflammatory lesions including pyogenic granuloma, peripheral ossifying fibroma, fibrous hyperplasia, peripheral giant cell granuloma, and in oral submucous fibrosis, oral leukoplakia and oral lichen planus. [3]

Oral Lichen Planus is probably the immunologically induced apoptotic result of basal keratinocytes due to cytotoxic $CD8^+$ response on modified keratinocytes surface antigen. Oral mucosal T lymphocytes interact in bidirectional manner in maintaining pathogenesis and chronicity of the lesion.[4]

Mediators of mast cells like prostaglandins and leukotrienes are effective secretagogues for the mucous and serous cells. This could attribute to increased saliva formation in OSMF. The effect of such chemical mediators explains the considerable histopathological changes in OSMF. Histamine, one such mediators probably attribute to edema in submucosal areas seen in early stages of OSMF. As vasopermeabilityis increased, eosinophilic chemotactic factor is released from the Mast Cells. This could possibly attribute to thepresence of eosinophils that are sometimes part of the inflammatory cell infiltrate, noticed in the early stages of OSMF. Interleukin-1 from the mast cells causesincreased fibroblastic response. Tryptase derived from mast cells causesamplified production of type-1 collagen and fibronectin thus attributing to increased fibrosis.[5]

Mast cells can be stained using metachromatic methods such as Toludine Blue O, Methylene Blue and Giemsastain. Other techniques include copper phthalocyanines like alcianblue with neutral red and alcian blue with safraninO.[6]The use of dyes that chelate cationic metals have also been reported. It is been observed that ferroin stain mast cells bright orange with great specificity and allows different cytoplasmic and nuclear stains for counterstaining.[6]

Heparin, a heteroglycan rich in half-sulfate esters present in Mast cells is responsible for metachromasia. TB is a small weakly hydrophilic cationic dye when attached to DNA or RNA, in chromatin or Nissl substance, stains blue whereas whenattached to glycosaminoglycans, granules of mast cell or cartilage matrix, itdisplays a purple metachromatic color. TB is applied from weakly acidic aqueous solutions. Most proteins are protonated and thus polycationic. Hence basic dye cations exchange with tissue cations that are associated with polyanions, known as basic dyeing. This process increases the chance of the system as proteins that are polycationic and are not associated with mobile cations, thus ion exchange cannot occur minimizing background staining and stain all forms of mast cells. [7]

Literature shows use of ToludineBlue for staining mast cells in oral lesions as a gold standard method. This is the first study to use Modified Ferroin Technique (MFT) for staining mast cells in oral lesions.

II. Materials and Methodology

Cases were retrieved from archives of Department of Oral and Maxillofacial Pathology, and were divided into three groups:

Group 1- (n=10) Oral Reactive lesions(ORL)
Group 2- (n=10) Oral lichen Planus(OLP)
Group 3- (n=10) Oral SubmucousFibrosis(OSMF)

Two sections of 4⌠m thickness from formalin fixed paraffin embedded tissues were obtained. One section was stained with ToludineBlue O and other with Modified Ferroin Technique.

Procedure for Modified Ferroin Technique:

Preparation of solutions:

Fast Green- 0.5g in 1000ml distilled water
1% acetic acid solution 1ml in 99ml distilled water
Safranin O- 0.19g in 1000ml distilled water

Procedure: Hydrate the slides using distilled water and stain the slides with Harris Haematoxylin for 10min followed by Fast Green for 5 min.Dip the slides in 1% acetic acid for 10-15 seconds and stain the slides with safranin O for 5 min. Dip the slides in 95% ethyl alcohol and mount the slides

III. Results

Modified Ferroin Technique stain for mast cells, a combination of phenonthroline and ferrous sulphate counterstained with light green produced intense orange color which contrasts the bluish violet nuclei of Mast cells.

Table1: Number of cases positive for mast cells

Cases	Toludine blue O	MFT
ORL	7	6
OLP	10	0
OSMF	10	0

Table 2: Mean of total number of mast cells present

Cases	Toludine blue O	MFT
ORL	9.6	10.5

OLP	11.1	0
OSMF	3.5	0

Statistical analysis was done, using Fischer Exact Test with $p<0.001$* making it statistically significant and Spearman Rank Correlation showing $r=0.375$ and $p=0.052$

IV. Discussion

Mast cells are considered to be multifunctional cells of immunity presenting themselves in several health and disease states. It is increasingly evident that maturation, function, and phenotype of mast cells are a direct result of the local microenvironment. It is also observed that they have a marked influence on their ability to particularly recognize and react to various stimuli by releasing an array of biologically active mediators.[1] In humans, reactive hyperplastic lesions are represented as most frequently encountered oral mucosal lesions.[8]Histopathology of oral reactive lesions shows neovascularization and inflammation depending on the phase of the lesion. Mast cells comprise of variable mediators within their granules. As mast cells are stimulated, they degranulate and bring about essential biological action.[2] FcεRI(high affinity Epsilon IgE receptor) initiates intracellular reactive oxygen species production. These will act as secondary messengers in a pathway of signal transduction leading to degranulation and cytokine synthesis .[9]

In our study 7/10 cases with Toludine Blue O and 6/10 cases with MFT were positive among Group 1, suggesting that it is only resonate to presume that the predominance of mast cell under degranulation would take place in a stage much before the active stimulation of angiogenesis and subsequent inflammatory reaction. This early stage could be defined as pre-inflammatory stage, prior to the active vascularity and increase in inflammatory cells.[3]

There is a growing awareness about thebidirectional interaction between oral mucosal T lymphocytes and mast cells, maintaining the chronicity and the pathogenesis of oral lichen planus. Mast cell-derived TNF-α activates T cells which further secretes RANTES and matrix metalloproteinase (MMP) causing continuous degranulation of mast cells, while MMPs prepare the endothelium and the adjacent connective tissue matrix for T cell migration.[10] Literature shows that intact mast cells significantly increased from normal mucosa to different grades of OSMF but degranulated mast cells significantly increased only in the very early and early stages of OSMF. On the contrary, in moderately advanced stage, mast cells decreased. It is also been suggested that inflammatory cells releasing cytokines could be a possible stimulant for the increase in the number of mast cells from normal to different grades of OSMF. [11]

In our study all 10 cases of OLP and OSMF stained for Toludine Blue O and contrarily none of the cases stained for MFT as it stains only degranulated mast cells(active form) indicating thatoral mucosal T lymphocytes interact in bidirectional manner thus maintaining the chronicity [4]and Interleukin-1 from the mast cell increases fibroblastic response along with Tryptase derived from mast cells, resulting in amplified production of type-1 collagen and fibronectin attributing to increased fibrosis, seen in increasing grades of the lesion respectively.[5]

Granules from rat peritoneal mast cells contained zinc:ironratioas 2:1 proportion and Copper being absent.Zinc is been easily released whereas Iron remains firmly bound. Iron may affect activity of mast cells directly via its redox properties.[12]Iron is a well-known quencher of free radicals and can be readily oxidized or reduced under conditions present within cells.

Phenanthrolines constitute heterocyclics as metal reagents in analytical chemistry. Ferroin is a metallochrome with o- phenanthroline. The complex contains tightly bound iron to nitrogen atoms of o-phenanthroline.[6]Ferroin has high staining affinity for mast cell granules indicating covalent bonding between carboxyl or sulphate groups of heparin and electrostatic bonding of ferroin.[6]Thus the negative charge density in heteroglycans are very large andhave increased capacity to bind water and counterions of ferroin.[6]

Ferroin is an iron binding REDOX indicatorthatbinds to granules of Mast cells and stain them intense orange. Iron may affect mast cell activity directly via its redox properties by stimulation of FcεRI, inducing the production of intracellular reactive oxygen species causing its degranulation. MFT stain degranulated mast cells(active form) while Toludine Blue stain mast cells of all groups.It is been observed that ferroin stain mast cells bright orange with great specificity and allows different cytoplasmic and nuclear stains for counterstaining whereas Toludine Blue is a small weak hydrophilic cationic dye, with minimal background staining and stain all forms of mast cells. MFT stain is also cost effective and less time consuming compared to Toludine blue.

V. Conclusion

Mast cells have gained a lot of importance in the recent years owing to vast number of chemical mediators are enigmatic, multifaceted protagonists of natural immunity as they release wide range of actions in many of the disease processes responding to IgE-dependent environmental antigens. Modified Ferroin Technique stain degranulated mast cells(active form) while Toludine Blue stain mast cells of all groups.

Modified Ferroin Technique stain helps in knowing the chronicity of lesion and thus aiding in the treatment planning for better diagnosis.The anti-mast cell therapy may offer an adjunct to the existing treatment modalities in the coming years as these cells havepotential to not only act as primary responders in pathology but also to react to changes in the environment by communicating with other cells implicated in physiology and immunology including innate, adaptive immunity and immune tolerance.

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TABLES:

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FIGURES:

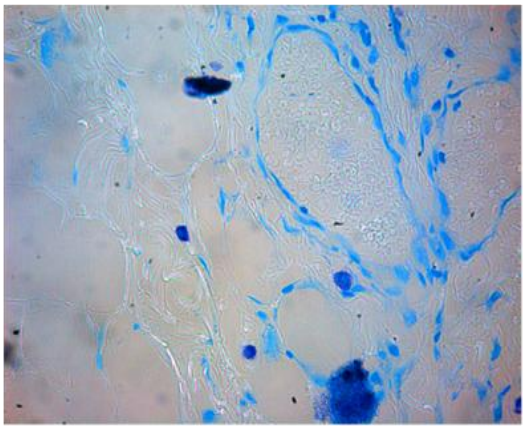

Fig 1

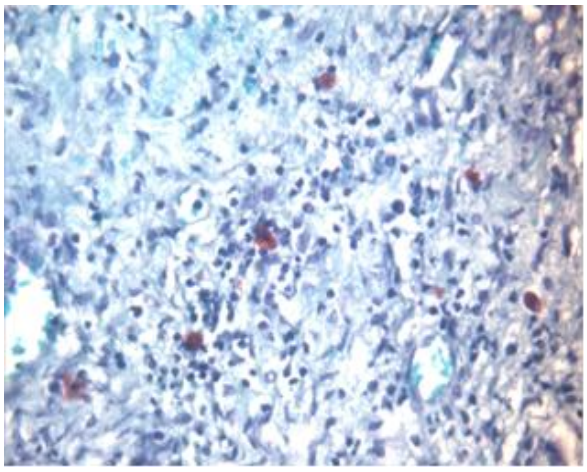

Fig 2

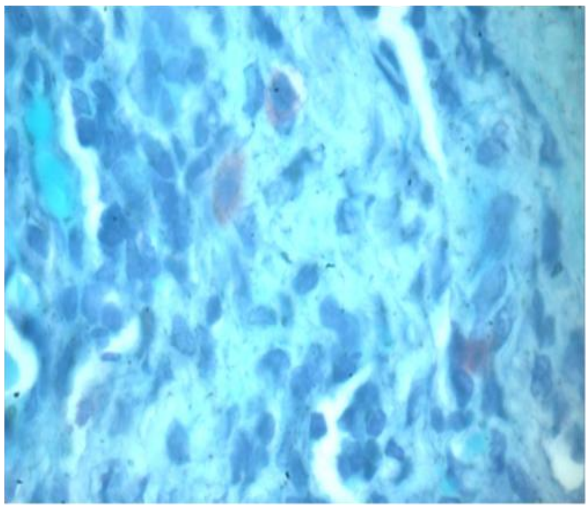

Fig 3

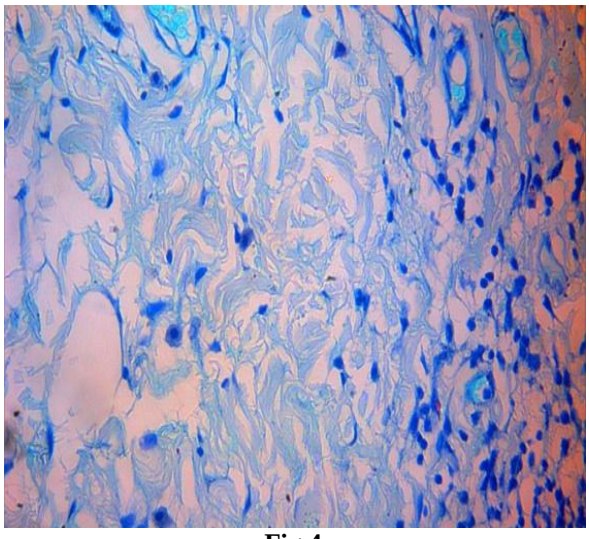

Fig 4

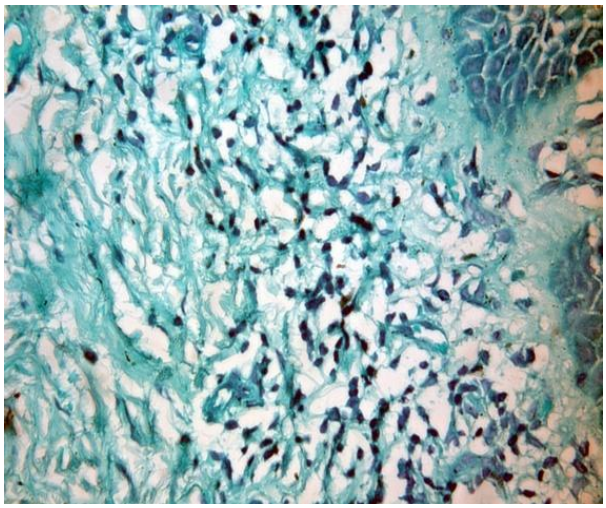

Fig 5

Fig 1: Positively stained mast cells using Toludine Blue O(40x) in Group 1
Fig 2: Positively stained mast cells using MFT(10x) in Group 1
Fig 3: Positively stained mast cells using MFT (40x) in Group 1
Fig 4: Negatively stained mast cells using MFT (40x) in Group 2
Fig 5: Negatively stained mast cells using MFT (40x) in Group 3

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