Original Article

Efficacy of Csaba stain to demonstrate mature and immature mast cells in oral inflammatory lesions: An *in-vitro* study

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Abstract

Background: Mast cells (MCs) are immune cells of the myeloid lineage and are present in connective tissues throughout the body. Mastocytosis regulates many physiologic processes and affects the pathogenesis of allergic conditions, anaphylactic reactions, autoimmune disorders and leukemias. Toluidine blue is commonly employed as a special stain for MCs that uniformly imparts blue color to both immature and mature cells. Csaba stain that distinguishes mature from immature MCs has not been widely explored. However, its use in differentiating mature and immature cells has not been reported in the literature. The identification of mature and immature MCs may provide a major clue for the diagnosis of any unrecognized systemic pathologies.

Objectives:

- To evaluate the staining efficacy of Csaba stain, toluidine blue and Leishman's stains in the identification of MCs
- To compare the effectiveness of Csaba stain, toluidine blue and Leishman's stains to identify MCs in inflammatory pathologic lesions.

Materials and Methods: Fifty sections of inflammatory lesions were subjected to Csaba stain, toluidine blue and Leishman's stains each for studying MCs. The staining efficacy of the three stains was compared for parameters such as homogeneity, specificity, staining intensity, granular staining property and differentiation between mature and immature cells.

Results: The Csaba, toluidine blue and Leishman's stains showed statistically insignificant $P = 0.50, 0.95, 0.29, 0.48$ for homogeneity, specificity, staining intensity and granular staining property respectively, but Csaba stain showed statistically significant $P = 0.0001^{*}$ in differentiating mature from immature cells.

Conclusion: Csaba stain is more effective in differentiating mature from immature MCs compared to the other special stains.

Keywords: Csaba stain, Leishman's stain, mature mast cells, metachromatic granules, toluidine blue stain

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INTRODUCTION

Routine histopathological examination involves hematoxylin and eosin (H and E) staining procedure for identification of normal structures and diagnosis of pathologies.[1,2] The H and E staining imparts blue color to the acidic components of tissues and varying shades of pink to the basic structures, mainly the cytoplasm of the cell, red blood cells, muscles, nerves and bone.[3] Diagnostic pathology poses challenges when routine stain demonstrates similar appearance for different tissue structures resulting in ambiguity to identify the tissue of origin. Various special stains are being employed at the pathology setup to distinguish different tissue structures to provide a clue to the diagnosis. Special stains such as Masson's Trichome and Van Gieson used for collagen detection, Carbol chromotrope and Congo red stains for eosinophils, Von Kossa for bone identification and Periodic Schiff stain for fungal organisms are commonly used in pathology laboratories that tries to resolve the diagnostic ambiguity in challenging cases.[4-6] Similarly, identification of mast cells (MCs) that play a vital role in body's defense mechanism is commonly demonstrated with the help of a differential stain, toluidine blue.[7-9]

MCs are essential for the immune system and production of hematopoietic stem cells. It develops from pluripotent bone marrow progenitor cells and stem cell factors in tissue microenvironment where it resides. Usually, MCs do not circulate in the blood vessels, but progenitors of MCs will move into the tissues and get differentiated into mature cells under the influence of various cytokines and stem cell factors [Figure 1]. MC granules are rich in heparin and histamine and therefore play a vital role in various physiological and pathological processes such as inflammation, oral lichen planus, oral squamous cell carcinoma, pyogenic granuloma and periapical cyst.[10-12] MCs get increased significantly in neurofibroma and usually provide a hint toward diagnosis.

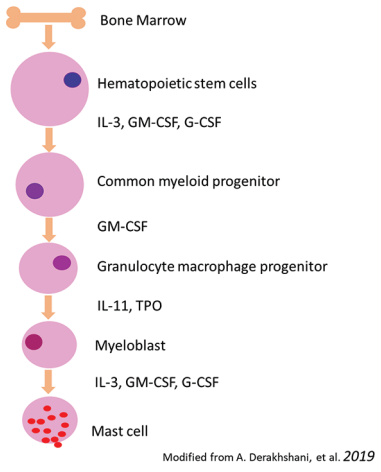

Figure 1: Differentiation stages of mast cells

Toluidine blue that is commonly used for the recognition of MC, stain the metachromatic granules observed in mature cells.[11] Stains such as Alcian blue, combination of Romanowsky stains namely Leishman, Alcian blue-pyronin Y, astra blue, azure II with Alcian blue, May–Grünwald Giemsa and Wright Giemsa have also been used to detect metachromatic granules of MC.[11,13] However, there is limited knowledge on the use of Csaba stain for the identification of MC. There is a need to know if Csaba stain could differentiate mature and immature MCs so as to assess the stage of differentiation of the disease process. Therefore, the present study aims to assess the staining efficacy of Csaba stain for mature and immature MCs and compare it with toluidine blue and Leishman's stain.

MATERIALS AND METHODS

Fifty paraffin-embedded tissue blocks were retrieved from the archives of the Department of Oral Pathology and Microbiology, Faculty of Dental Sciences, Ramaiah University of Applied Sciences, Bengaluru. The University ethical committee approval was obtained (EC-2021/F/055) was obtained. The oral pathologies that histologically showed marked inflammation in the lamina propria were included in the study. Three 4-µm sections were cut from each block, deparaffinized, rehydrated and stained with hematoxylin and eosin staining (Harris' Hematoxylin: H30271; Nice Chemicals Pvt. Ltd., Kochi, Kerala, India; eosin yellow stain solution – 2% W/V: E30971: Nice Chemicals Pvt. Ltd., Kochi, Kerala, India), Toluidine blue stain (M. S) (T32709; Nice Chemicals Pvt. Ltd., Kochi, Kerala, India), Leishman's stain (24SS717-75, ARKRAY Healthcare Pvt. Ltd, Surat, Gujarat, India) and Csaba stain [Ammonium ferric sulfate (iron alum): A12339; Safranine (M. S.): S20109, Nice Chemicals Pvt. Ltd., Kochi, Kerala, India; Alcian blue 8GX, Hi-Cert, RM471-5G, HIMEDIA, Nashik, India]. The standard protocol for H and E staining was followed.[14,15] The solutions for Csaba, Toluidine blue and Leishman's stains were freshly prepared at the time of staining.

The staining efficacy of Csaba stain, Toluidine blue and Leishman's stains was evaluated for studying MC. The staining efficacy of the three stains was compared in inflammatory lesions for the following parameters – homogeneity, specificity of staining, intensity, granule-staining property and differentiation between mature and immature cells.

Toluidine blue staining

Deparaffinized sections were placed in two changes of Xylene for 10 min each. They were hydrated through 70% and 60% alcohol for 5 min each. The sections were washed in running tap water for 5 min and placed in freshly prepared toluidine blue solution for 2–5 min. The slides were rinsed in water followed by 95% alcohol for two changes. The sections were blot dried, cleared in xylene and mounted using DPX (Nice Chemicals Pvt. Ltd., Kochi, Kerala, India).

Leishman's staining

Deparaffinization of the slides was carried out on a hot plate and then subjected to two changes of xylene for 10 min each. The slides were hydrated through 70% and 60% alcohol for 5 min and washed in tap water for 5 min. The tissues were covered with Leishman's stain for 2 min, rinsed in water, followed by blotting, drying and mounting in DPX.

Csaba staining

Deparaffinized sections were place in two changes of xylene for 10 min each. The tissue sections were passed through 70% and 60% alcohol for 5 min each. The sections were washed in running tap water for 5 min and then placed in the prepared Csaba solution for 5–8 min. Finally, the sections were rinsed in tap water, followed by 1 dip in 1% absolute alcohol. The stained sections were blotted, cleared in xylene and mounted using DPX.

Evaluation of staining

The stained slides were evaluated by two oral pathologists for the following parameters. The staining was considered homogeneous when uniform staining of all MC was observed throughout the section. The intensity of staining was assessed by observing the contrast of color with the surrounding structures using the 40× objective. It was scored as 1 – mild; 2 – moderate; 3 – intense. The next parameter that was considered was specificity that assessed how best a particular stain could be localized only to MC and their granules, and was graded as specific and nonspecific staining. Granular staining property was categorized into distinct or indistinct based on the appearance of prominent granules in the stained tissue sections. The property of each stain to distinguish mature from immature MC was also assessed.

Statistical analysis

The statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) software (IBM SPSS Statistics for Windows, version 20.0 [IBM Corp., Armonk, NY, USA]). Comparison between the study groups was performed using Chi-square test. Values of $P \leq 0.05$ were considered statistically significant.

RESULTS

Out of the fifty cases that were analyzed, homogeneity in staining was observed in 42 (84%), 41 (82%) and 45 (90%) cases each for Csaba, toluidine blue and Leishman's stains, respectively, with a *P* value of 0.5. Distinct granular staining property was evident in 41 (82%), 36 (72%) and 39 (78%) cases each ($P = 0.485$) for the above special stains, respectively, suggesting that the three stains are equally effective for enhancing the granules [Table 1].

Specificity of the stains to impart staining only to MC was prominently appreciated in 42 (84%) cases each of Csaba and Leishman's stains and 41 (82%) cases of toluidine blue staining with a *P* value of 0.953. The staining intensity was found to be intense in 42 (84%) cases each for Csaba and toluidine blue and 43 (86%) cases of Leishman's-stained sections. Moderate intensity was observed in 7 (14%), 8 (16%) and 4 (8%) cases and mild in 1 (2%), 0 and 3 (6%) cases each for Csaba, toluidine blue and Leishman's stains, respectively, with a $P = 0.299$ [Table 1].

The differential property of MC to impart different colors to mature and immature MCs was assessed as an additional parameter. It was observed that out of the fifty cases that were stained, a combination of mature and immature cells was appreciated in 13 cases of Csaba-stained sections.

Table 1: Comparison of the efficacy of Csaba, Toluidine blue and Leishman's stains to demonstrate mast cells

Parameters	Parameters sub-division	Csaba stain (*N*=50), *n* (%)	Toluidine blue (*N*=50), *n* (%)	Leishman's stain (*N*=50), *n* (%)	*P*
Homogeneity	Homogenous	42 (84)	41 (82)	45 (90)	0.500
	Nonhomogenous	8 (16)	9 (18)	5 (10)	
Specificity	Defined	42 (84)	41 (82)	42 (84)	0.953
	Variable	8 (16)	9 (18)	8 (16)	
Staining intensity	Intense	42 (84)	42 (84)	43 (86)	0.299
	Moderate	7 (14)	8 (16)	4 (8)	
	Mild	1 (2)	0	3 (6)	
Granular staining property	Distinct	41 (82)	36 (72)	39 (78)	0.485
	Indistinct	9 (18)	14 (28)	11 (22)	

*$P<0.05$, statistically significant. *N*: Total samples used in the study, *n*: Number of samples positive for each parameter

Such a difference was noted neither in toluidine blue nor Leishman's-stained sections. When this parameter was compared between Csaba and toluidine blue staining, there was a statistically significant difference with a $P = 0.0001$ [Figure 2 and Table 2]. Similar significant difference was observed on comparison of this parameter between Csaba- and Leishman's-stained sections of inflammatory lesions.

DISCUSSION

The present study was conducted to analyze the staining efficacy of Csaba, Leishman's and Toluidine blue to identify MC. The parameters that were considered for analysis included homogeneity, specificity, staining intensity, granular staining property and maturity of MC. The lesions that were included in the study were inflammatory/reactive lesions, such as oral lichen planus, pyogenic granuloma, inflammatory hyperplasia and granulation tissue. The commonly employed special stain for detection of MC available in the literature is the toluidine blue.[16] There has been minimal research carried out on the use of Csaba and Leishman's stains for the identification of MC till date. As Csaba and Leishman's stains can be easily prepared in a routine histopathology laboratory, the present study aimed to assess their efficacy in staining MC.

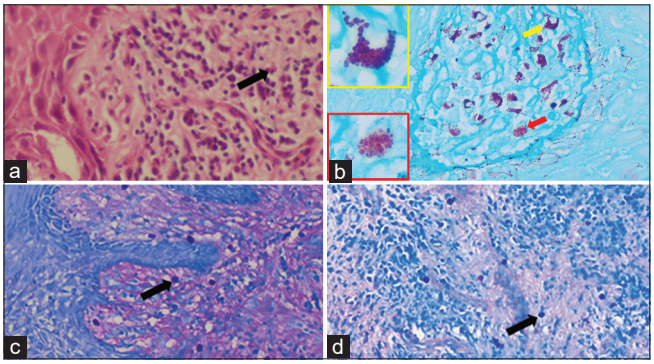

Figure 2: Hematoxylin and eosin, Csaba, toluidine blue and Leishman's staining for mast cells. (a) Mast cells (black arrow) in routine staining poses difficulty in identification (H and E, ×100); (b) Mature mast cells (red arrow) stained reddish pink, Inset with red border (×1000) and immature cells (yellow arrow) showing blue colored granules , Inset with yellow border (x1000) (Csaba stain, ×400). Background tissue is masked highlighting only the mast cells. (c) Uniform staining of all mast cells (black arrow) with difficulty in differentiating mature from immature mast cells (toluidine blue stain, ×200); staining of mast cells (black arrow) observed using Leishman's stain, ×200

Table 2: Differentiation between mature/immature cells observed among the study groups

	Appreciated	Not appreciated	Total	*P*
Csaba stain	13	37	50	0.00011*
Toluidine blue	0	50	50	
Csaba stain	13	37	50	0.00011*
Leishman's stain	0	50	50	

*$P<0.05$, statistically significant

The homogeneity, granular staining property, specificity and staining intensity of MC were similar in Csaba, Leishman's and Toluidine blue stains, suggesting that the three stains are equally effective in the identification of granules and the MC have uniformly taken up the stain[16,17] [Figure 2 and Table 1]. Narayan *et al.* estimated the mean MC count in different grades of oral squamous cell carcinoma and have also found toluidine blue to be effective in identification.[18]

As Leishman's stain belongs to the Romanowsky group that stain the metachromatic granules prominently, it was included in the present study. Studies that have used Leishman's stain to detect MC in human oral tissues are rarely reported in the literature. However, Leclere *et al.*, 2006, have demonstrated that toluidine blue was effective in the identification of metachromatic granules of MC in equine bronchoalveolar lavage fluid.[9]

The unique feature noted specifically in Csaba-stained slides is that 13 cases showed a clear distinction between immature and mature cells, which was not evident in either Leishman's or toluidine blue stains. The immature cells were blue, whereas the mature cells were stained with red. Differences in the content of sulfated proteoglycans, showed differences in the staining of MC varying from red to blue or mixed red and blue colors in Csaba stain. Therefore, cells that are Alcianophilic are blue, representing immature cells, and those that are red are Safraninophilic and denote mature cells.

In human bone marrow, there are four different morphological types of MCs based on their maturation stages. They include the tryptase-positive nongranulated blast cell, the metachromatic blast cell, atypical MC type II/the promastocyte and the mature MC [Figure 1]. The immature forms of MC are predominantly found in MC leukemia, acute and chronic myeloid leukemias and other myelodysplastic disorders. However, the mature MCs are significantly enhanced in allergic disorders, anaphylactic reactions, normal tissue remodeling and wound healing processes, angiogenesis and autoimmune disorders such as pemphigus, pemphigoid, urticaria, vasculitis, insulin-dependent diabetes mellitus and rheumatoid arthritis.[19-21] Increase in the number of MCs called as mastocytosis can be detected using skin and bone marrow biopsies. As MCs are rich in tryptase, mastocytosis can also be assessed by estimating the serum tryptase levels.[19]

There are very few studies documented so far in the literature that have employed Csaba stain for the detection of MC in oral lesions. Tan *et al*. (2004) have demonstrated MC in various stages of hemangioma using Csaba stain and the immunostains-Clusterin/apolipoprotein J.[21] They have suggested that the MC that show positivity for Csaba stain are of biogenic amine phenotype, predominantly comprising histamine. However, their study lacks differentiation between mature and immature cells. The present study has very convincingly demonstrated the use of Csaba stain to distinguish mature from immature MCs [Figure 2 and Table 2].

CONCLUSION

The present study showed that Csaba, Leishman's and Toluidine blue stains are equally efficient in identifying the MC in inflammatory lesions. The novelty of the present study lies in demonstrating different staining characteristics of mature and immature MCs using Csaba stain, thereby helping in distinguishing the types of MC.

A lot of literature evidence suggests that toluidine blue can be used as a special stain for the recognition of MC. Mastocytosis can be diagnosed using differential stains for MC. Increase in immature MCs has been predominantly found in MC leukemias, acute and chronic myeloid leukemias and other myelodysplastic disorders, which fails to be differentiated through the common special stains. The present study is the first of its kind in employing a novel stain, Csaba stain, to distinguish mature from immature MCs. This distinction not only signifies the differentiation stage of MC but also provides an important clue to the clinician to venture into further investigations for the diagnosis of underlying systemic pathologies.

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Conflicts of interest
There are no conflicts of interest.

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