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Original Research Article

Mast Cell Characterization and Density in Normal and Abnormally Invasive Placental Tissue Using Five Histochemical Stains

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ABSTRACT

Mast cells (MCs) are multifunctional immune cells implicated in tissue remodeling and immune regulation. Yet, their densities and distribution in human placental tissues, particularly in cases of abnormal placental invasion such as placenta accreta spectrum (PAS), remain underexplored. While immunohistochemistry is considered the gold standard for MCs identification, it is resource-intensive, requiring specialized skills, making histochemical staining a practical alternative. Limited studies have provided conflicting results on the most effective histochemical stains for identifying MCs in human placental tissues.

This study aimed to evaluate mast cell density and characterize their histochemical staining properties in placental tissues from control and experimental groups. The control group comprised five normal full-term placentas obtained from vaginal deliveries, while the experimental group included five placentas from cases of placenta accreta spectrum (PAS) delivered by abdominal hysterectomy. Additionally, the study sought to identify the most effective histochemical stain for mast cell identification and characterization

Five histochemical stains; Toluidine Blue, Periodic Acid-Schiff, Giemsa, Hematoxylin & Eosin (H&E), and Alcian Blue with Safranin O were used to stain sections from each placental sample of the control and the experimental groups. MCs were counted in 10 fields at ×400 magnification for each section, and their densities were compared between the control and the PAS cases for all stains used. Statistical significance was set at $p = 0.05$.

Toluidine Blue enabled the highest mast cell counts, outperforming other stains in identifying and characterizing mast cells. Morphologically, three types of mast cells were recognized according to their granular content. Mast cell densities were significantly higher in placenta accreta cases compared to the control, suggesting a potential role for MCs in the pathogenesis of PAS.

In conclusion, this study highlights the value of histochemical staining—particularly Toluidine Blue—in reliably identifying mast cells within placental tissue. The findings suggest a possible involvement of mast cells in the pathogenesis of placenta accreta, warranting further research into their underlying mechanisms and their potential utility as diagnostic markers or therapeutic targets

KEYWORDS: Mast cells, Placenta Accreta Spectrum, Histochemical staining, Toluidine Blue.

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INTRODUCTION

Placenta Accreta Spectrum (PAS) is a serious obstetric complication characterized by a bnormal placental invasion into the myometrium, leading to increased maternal morbidity and mortality. The condition includes placenta accreta (anchoring villi adhere to the superficial myometrium without interposing decidua), placenta increta (the placental villi penetrate into the myometrium), and placenta percreta (anchoring villous tissue penetrates through the entire uterine wall even to the surrounding organs), depending on the depth of trophoblast invasion [1]. PAS is associated with excessive hemorrhage during delivery, often necessitating hysterectomy, and poses significant challenges in maternal-fetal medicine [2]. The underlying pathophysiology of PAS remains incompletely understood, despite advancements in its imaging and surgical management [3]. The pathogenesis of PAS is complex and multifactorial, involving elements such as dysregulated angiogenesis, defective decidualization, and abnormal trophoblast invasion [4]. MCs are known to contribute to processes like angiogenesis and tissue remodeling, which are relevant to placental development [5]. However, direct evidence linking MCs to PAS pathogenesis is currently limited.

MCs are immune cells of myeloid lineage involved in various physiological processes, including homeostasis, tissue inflammation and repair, innate and adaptive immunity [6], immune tolerance, and host defense against pathogens [7]. These cells also play a crucial role in the immunopathology of both immediate and delayed hypersensitivity reactions [8]. MCs are large, granulated cells, widely distributed in vascularized tissues, where their maturation, phenotype, and function are influenced by the local microenvironment [9].

MCs express multiple surface receptors, and they produce and store numerous mediators, which are released upon their activation in response to a variety of internal and external stimuli, triggering diverse responses in surrounding tissues [10]. MCs' mediators include biogenic amines (histamine, serotonin, dopamine), mucopolysaccharides (heparin, chondroitin sulfate), proteoglycans (serglycin), proteases (tryptases, chymases, carboxypeptidase A), cytokines, chemokines, growth factors, hormones, and other bioactive compounds [11,12,13].

Histochemical staining is widely used to detect MCs on histological specimens, since it is relatively simple, low-cost, efficient, and is applicable to nearly any human and animal tissues. The secretory granules of MCs are rich in sulfated glycosaminoglycans (proteoglycans), primarily heparin, which facilitates the binding of dye molecules and enables selective imaging of these cells through histochemical staining [14,15].

While Toluidine Blue (TB) is often considered the most effective for mast cell (MC) identification due to its strong metachromatic staining of MC granules [16], conflicting data from histochemical and comparative studies highlight that the effectiveness of TB can vary depending on tissue type, fixation, and mast cell subtype. Comparative studies have shown that Giemsa and Alcian Blue–Safranin staining may provide better accuracy, especially when mast cell activation status, subtype differentiation, or functional assessment is required [17]. In this study, we evaluated the effectiveness of five different histochemical stains in identifying MCs in placental tissue from control and experimental groups.

METHODOLOGY

This study was conducted on formalin-fixed paraffin-embedded tissue samples from human placental tissues, in accordance with the human study protocol approved by the Ethical Review Committee of Baghdad University, Baghdad, Iraq.

Human placental tissues were collected from five cases of placenta accreta spectrum (PAS) delivered by abdominal hysterectomy at or near term (experimental group), and from five normal full-term placentas obtained following uncomplicated vaginal deliveries (control group). All samples were collected by a

consultant gynecologist at Baghdad Teaching Hospital, Medical City, Bab Al-Moatham, Baghdad, Iraq.

Full-thickness placental parenchymal samples were taken from five marginal areas and one central region of the placental disc. Tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin, yielding six tissue blocks per patient.

From each tissue block, five sections (4 µm thick) were cut, deparaffinized with xylene, rehydrated through graded ethanol solutions, and stained using the following techniques:

· Hematoxylin & Eosin (H&E) staining was performed following the protocol by Bancroft and Gamble, using reagents from Nice Chemical Pvt. Ltd. (India). [18].

· Giemsa staining was performed according to the method described by Leclere et al., using reagents from Himedia Pvt. Ltd. [19].

· Periodic Acid-Schiff (PAS) staining was performed following the modified McManus technique. [20].

· Alcian Blue & Safranin O staining was carried out following the method of Enerbäck, using Alcian Blue from Hopkin & Williams, England, and Safranin from Areen Alashhab Medical Co., Iraq. [21], [22].

· Toluidine Blue staining was performed as per Romeis' standard protocol, using 1% toluidine blue in 0.5 M HCl (pH 0.5) from Himedia Pvt. [23].

All staining solutions were freshly prepared. After staining, sections were dehydrated through graded alcohol, cleared in xylene, and mounted with DPX (Spectrum Reagents and Chemical Pvt. Ltd., India). Images were captured and processed with A33.1502 - 9.7" LCD Digital microscope, 5.0 M, Android Pad Camera, and image processing software (OPTO-EDO CO., LTD, Beijing, China). Images were saved as JPEG files and processed in PhotoImpact 3.0 (Ulead Systems, Inc., CA) at 300 DPI resolution.

Staining intensity was assessed subjectively by comparing mast cells to the surrounding connective tissue. We looked for color differences (e.g., blue mast cells against a pinkish background) and cell distribution patterns (localized clusters of mast cells). A score system was applied as follows: 1 - Mild/Poor, 2 - Moderate, 3 - Strong/Good. The authors assessed the histological evaluation of all stained sections for the presence of MCs. Large cells with distinct borders and central or slightly eccentric nuclei with clumped chromatin, and containing moderate-to-abundant amounts of small, purple-colored granules were identified as mast cells. Degranulated cells were not counted as mast cells unless most of the granules remained within their cytoplasm. Cells containing variable-sized granules and moderate to large-sized vacuoles were classified as macrophages.

For each tissue block, 5-10 sections were prepared. MCs' numbers were determined subjectively by counting positively stained cells in ten selected fields of view at 400× magnification per section. The stained sections were scanned under low-power magnification first to determine the general architecture of the slide, and then the representative fields were selected carefully in each slide by scanning the slide from left to right to avoid recounting the same areas. Five of these fields covered the chorionic villi (fetal side of the placenta) and five covered the decidua /myometrium (maternal side of the placenta). To ensure accuracy, mast cells were counted on serial sections for comparison across different staining methods. All statistical analyses were performed using SPSS Software version 20. A p-value $\leq$ 0.05 was considered statistically significant.

RESULTS

In this study, we evaluated the applicability of five distinct histochemical staining techniques for characterizing mast cell populations in human placental tissue, focusing on two key aspects: their morphological features and their density within the tissue.

Mast cells Morphology: In H&E-stained tissue sections, MCs appeared as large, pale pink, round, spindle, or polygonal-shaped cells with moderately abundant cytoplasm and visible granules that are weakly stained. They displayed a round-to-oval nucleus with

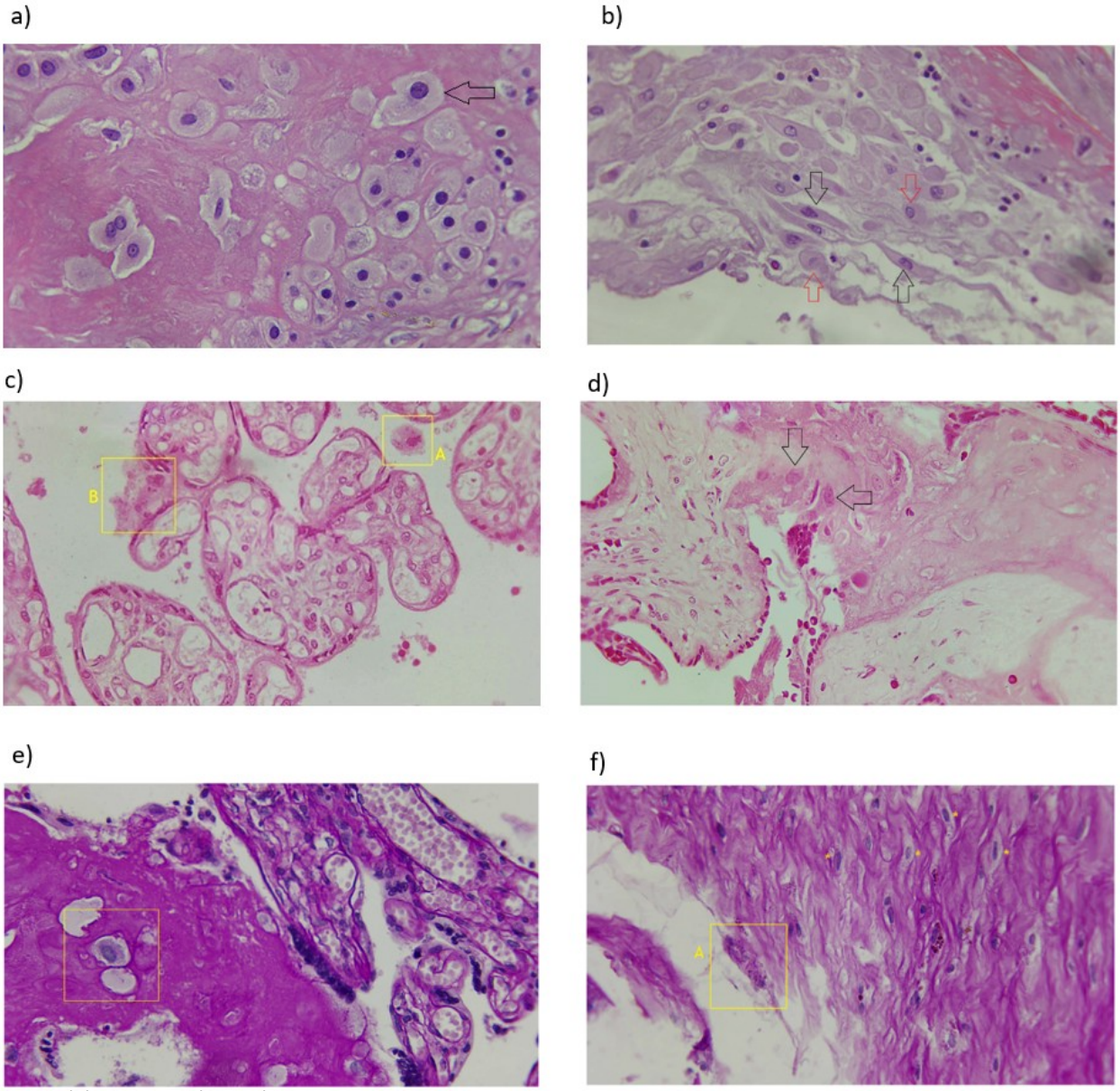

Fig. 1: (a) Mast cell (arrow) seen as large rounded cell with abundant pale-pink granular cytoplasm in decidua of placenta accreta group. H&E, X400. (b) Spindle shaped MCs (black arrows) and round shaped decidual cells (red arrows) shown in decidua of placenta accreta group. H&E, X400. (c) Resting oval mast cell {A} and degranulating mast cell {B} shown in the intervillous spaces of placenta accreta group. Giemsa stain, X400. (d) Active MCs with increased granular density in decidua of placenta accreta group are arrowed. Giemsa stain, X400. (e) Resting round shaped mast cell (highlighted in the box) with magenta staining cytoplasmic granules in decidua of placenta accreta group. Periodic Acid Schiff stain, X400. (f) Degranulating mast cell {A} and the multiple dispersed spindle-shaped mast cells (*) in decidua of placenta accreta group. Periodic Acid Schiff, X400. In the Alcian Blue & Safranin-stained sections, MCs were identified relatively easily against the orange/ red background from safranin, which stains other tissue elements (Fig. 2a). Moreover, two MC subtypes were distinguished: connective tissue-type mast cells (CTMCs), which stained red (safranin-positive), and mucosal-type mast cells (MMCs) that stained blue (Alcian blue-positive). (Fig. 2b).

clumped chromatin and indistinct or no nucleoli (Fig. 1a). The spindle-shaped MCs were difficult to differentiate from fibroblasts. Their larger size and darkly stained granules are distinguishing factors (Fig. 1b). In Giemsa-stained tissue sections, resting MCs appeared as large, rounded cells with blue nuclei and reddish-purple cytoplasmic granules (Fig. 1c). In contrast, active MCs revealed increased granular density (Fig.1d). In Periodic Acid Schiff-stained sections, MC granules showed weak magenta staining (Fig. 1e). The background structures, such as glycogen-rich areas or mucins of the decidua, appeared bright magenta, helping to outline other cell types nearby (Fig. 1f).

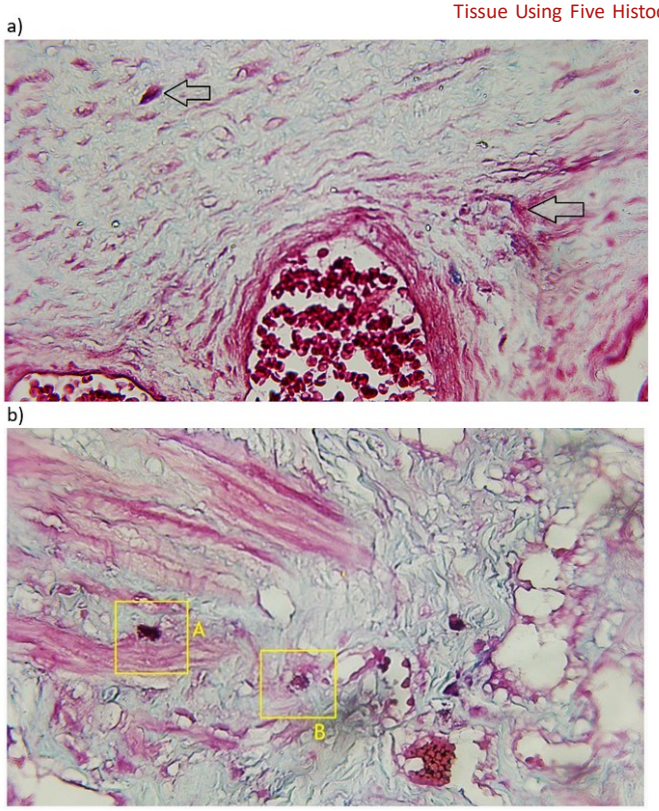

Fig. 2: (a) Mast cells (arrows) in the decidua of the placenta accreta group. Alcian Blue & Safranin stain, X400. (b) Blue-stained MMC type {A} and red-stained CTMC type {B} are shown in the placenta accreta group. Alcian Blue & Safranin stain, X400.

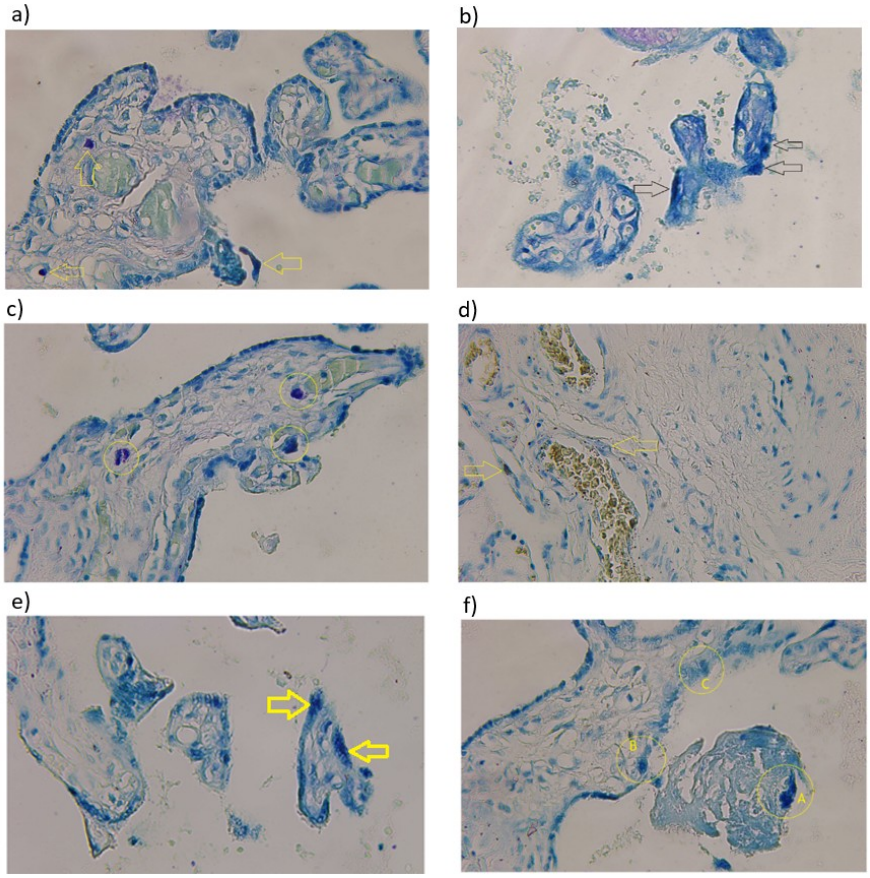

Fig. 3: (a) Mast cells with blue nuclei and purple granular cytoplasm (arrowed) in the fetal part of the placenta accreta group. Toluidine blue, X400. (b) Activated MCs with enhanced staining (arrows) in perivillous areas of terminal chorionic villi in the placenta accreta group. Toluidine blue, X400. (c) Mast cells (encircled) in intermediate chorionic villi of placenta accreta group. They appear as rounded cells with dense purple granules in their cytoplasm, obscuring their nuclei. Toluidine blue, X400. (d) Spindle-shaped MCs in the perivascular regions of the placenta accreta group. Toluidine blue, X400. (e) Hypogranular MCs with diffuse cytoplasm (arrows) in the chorionic villi of the normal placenta group. Toluidine blue, X400. (f) The intensely stained mast cell (A), the mast cell with vacuolization and cytoplasmic processes (B), and the mast cell with decreased granular content and faint metachromatic staining (C) are shown in chorionic villi of the normal placenta group. Toluidine blue, X400.

The staining intensity for each of the five staining techniques was evaluated as the following: the staining intensity score for both Toluidine blue and Alcian blue & Safranin stain is 3 (Strong/Good), for PAS and Giemsa is 2 (Moderate), and for H&E is 1 (Mild/Poor). The greatest contrast of MCs with connective tissue background in placental tissue was found in Toluidine blue and Alcian Blue & Safranin stained sections, followed by those stained with Periodic Acid Schiff, Giemsa, and H&E in decreasing order.

Mast cell density: For each studied case of the control and experimental groups, the mean number of MCs per 10 fields of view (X400) was counted in the five different histochemical stains for each case of the placenta accreta group and the control group. Respective p-values obtained from statistical comparisons between MC numbers in placenta accreta cases and the normal control for each stain were found using t-tests. For each stain, the aggregated mean and SD for the entire placenta accreta group were compared with the aggregated mean and SD for the whole control group, and the p-values were calculated. *P*-values of <0.05 were regarded as statistically significant (Table 1).

As shown in Table 1, for all the placenta accreta cases, Toluidine blue detected the largest mean number of mast cells (44.82), followed by Alcian blue & Safranin (30.62), Periodic Acid Schiff (27.92), Giemsa (26.60), and H&E (19.78). The differences in the mean number of the identified MCs in the individual placenta accreta cases compared to that of the normal placenta of the control cases, were statistically significant for Toluidine Blue *(P* = 0.0038) and A & S (*P* = 0.0193), only. Whereas PAS (*P* = 0.0826), Giemsa (*P* = 0.1486) and H&E (*P* = 0.0873) stains showed near-significant differences (0.05 < P < 0.1) suggesting possible differences, but they are not statistically significant at the 0.05 threshold.

Table 1: Mast Cell Counts in Placental Sections across Stains.

Stain	Group	mean ± SD	Mean Difference	t-statistic	P-value
H & E	PA (n=5 cases)	19.78 ± 4.18	2.58	0.0807	0.0873
	Control (n =5 cases)	17.2 ± 3.42			
Giemsa	PA (n=5 cases)	26.6 ± 5.33	3.6	0.1315	0.1486
	Control (n =5 cases)	23 ± 4.58			
PAS	PA (n=5 cases)	27.92 ± 5.01	5.36	0.0864	0.0826
	Control (n =5 cases)	22.56 ± 3.6			
A & S	PA (n=5 cases)	30.62 ± 8.12	9.64	0.0244	0.0193
	Control (n =5 cases)	20.98 ± 20.98			
Toluidine blue	PA (n=5 cases)	44.82 ± 6.93	12.44	0.0027	0.0038
	Control (n =5 cases)	32.38 ± 5.6			

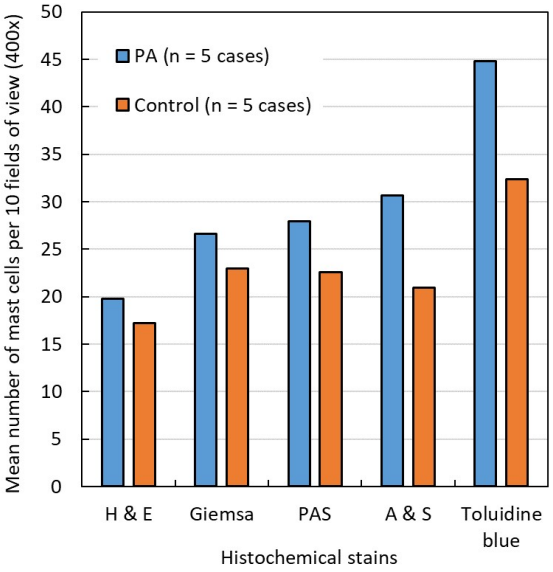

Fig. 4: Histogram of mean mast cell number identified per 10 fields of view (400x) in both placenta accreta group (blue) and control group (yellow) cases as revealed by five different histochemical stains.

A comparison between the mean mast cell density in placenta accreta cases and in control cases, as counted in all five histochemical stains, is shown in the histogram (Fig. 4).

DISCUSSION

MCs are characterized by their cytoplasmic granules, which contain proteoglycans (PG) and bioactive mediators such as histamine, heparin, tryptase, and chymase. These granules exhibit metachromasia— a unique staining property where they appear in a different color than the dye used. This feature allows MCs identification using specific histochemical stains [24].

In addition, the protease content of MC granules is crucial for their detection through immunohistochemical (IHC) methods, using monoclonal antibodies targeting these proteases, including tryptase, chymase, and carboxypeptidase A [25].

While IHC provides a precise way of identifying MCs, it is expensive, time-consuming, and requires specialized laboratory expertise, making histochemical staining a more practical option for MCs identification in many settings.

Our findings demonstrated that Toluidine Blue and the combined Alcian Blue & Safranin stains were the most effective in providing clear visualization of MCs and allowing for differentiation of subtypes, making them a valuable histological stain for MCs identification. Giemsa and PAS staining showed moderate efficacy, while Hematoxylin and Eosin (H&E) was the least effective in identifying MCs due to its poor granule contrast.

In the Toluidine blue sections, MCs were easily identified within the placental tissue as large oval or spindle-shaped cells with a cytoplasm that contained blue nuclei and purple granules. The background connective tissue was stained in shades of blue.

These staining characteristics are consistent with previous studies reporting that Toluidine blue effectively highlights mast cell granules through metachromatic staining, aiding their identification within various human tissues [26-28].

However, as shown by [29], the metachromatic effectiveness of Toluidine Blue diminishes in degranulated or immature MCs, leading to under-detection in certain contexts.

The combined Alcian blue and Safranin staining method gave a clear overall context of the placental architecture as it highlighted both acidic mucins (blue) and neutral components (orange/red). This dual staining method also effectively distinguished MCs subtypes, with connective tissue-type mast cells (CTMCs) staining red (safranin-positive) and mucosal-type mast cells (MMCs) staining blue (Alcian blue-positive).

This pattern of differential staining is consistent with previous findings by [28], who demonstrated that mast cell subtypes exhibit distinct affinities for these dyes based on their proteoglycan content. This is particularly relevant for placental tissue, where MCs may exhibit functional heterogeneity depending on their localization within the tissue.

Giemsa stain produced a purplish-red staining of MCs by binding metachromatically to the acidic components of their granules (heparin and histamine). Giemsa stain allowed for moderate identification of MCs, and in line with its reputation as a special MC stain, it provided a strong contrast between MCs and the background tissue [18].

Periodic acid Schiff (PAS) stain is often used to highlight structures containing high amounts of carbohydrate molecules [30]. The periodic acid component of this stain oxidizes the hydroxyl groups of adjacent sugar molecules to produce aldehydes, which then attach to the Schiff reagent, forming a red magenta color that helps to visualize glycogen, glycoprotein, glycolipids, and mucins in tissues [31].

Although the Periodic Acid-Schiff (PAS) stain is not routinely used for identifying MCs, it can react with the polysaccharides and glycoproteins within MC granules, producing magenta-colored granules. In this study, the periodic acid schiff staining method offered valuable insights into the placental structure and its cellular components, though it provided less contrast compared to Toluidine Blue or A&S. These findings align with previous studies that

Table 2: The staining characteristics of mast cells using different histochemical stains.

Stain	Purpose	Mast Cell Appearance
Hematoxylin and Eosin (H&E)	General tissue architecture visualization	Large, pale pink, round or spindle-shaped cells with abundant cytoplasm and weakly stained granules
Giemsa	Identifies mast cells via metachromatic granules	Granules stain purplish-red
Periodic Acid-Schiff (PAS)	Detects polysaccharides and mucosubstances	Granules stain magenta due to polysaccharides and glycoproteins
Alcian Blue & Safranin	Differentiates mast cell subtypes	CTMCs stain red (Safranin-positive), MMCs stain blue (Alcian blue-positive)
Toluidine Blue	Binds to glycosaminoglycans in granules (metachromatic stain)	Granules stain purple or violet; background stains blue

have utilized PAS staining to examine human placental tissue [32].

H&E staining method offered limited visibility of MCs which were often indistinct from the surrounding pink-stained connective tissue and their granules stained weakly. This finding is consistent with previous studies that considered H&E unreliable for MCs identification in tissue samples [33].

A summary of the main staining characteristics of MCs is given in Table 2.

The detectability of MCs with the common metachromatic stains significantly depends on factors such as: type and maturity of MCs, the test tissue type, the dye used, incubation solution pH, and the staining duration, fixation solution type, fixation time, and the final processing technique of stained preparations [34,35]. In addition to assessing staining efficacy, we also evaluated MCs' morphology and distribution within the placental tissue.

In this study, several morphological forms of MCs have been recognized with various degrees of clarity in the placental tissue of both study and control groups, mainly in the Toluidine blue-stained sections. MCs appeared in both round and spindle-shaped forms, with variations in their cytoplasmic granule density and staining patterns. Accordingly, we could morphologically classify MCs in placental tissue into the following forms depending on their granular content:

Granulated MCs - Round or oval cells with densely packed metachromatically stained granules, typically found within chorionic villi stroma or decidua. These are intact cells that functionally represent quiescent or resting MCs, in a storage mode, ready to be activated [36].

Hypogranulated or Partially Degranulated MCs - Elongated cells with cytoplasmic processes and variable granule content. They display reduced granule density, showing vacuolization or diffuse cytoplasmic granule distribution indicative of partial activation. These cells are commonly located near fetal vessels in the chorionic plate or decidual arterioles and possibly represent an activated or tissue-resident phenotype; potentially involved in matrix remodeling, angiogenesis, or immune cell recruitment [37].

Their perivascular location suggests possible interactions with endothelial cells and perhaps a role in vasomodulation or immune signaling [38].

Degranulated or empty MCs – Cells with reduced or absent granules, found in regions of high inflammatory or immune activity, such as areas near trophoblast invasion or spiral artery remodeling. These are the fully activated MCs that have undergone excessive degranulation and show a clear cytoplasm, which is devoid of granules, displaying a "ghost cell" appearance [39].

These morphological differences may reflect

Table 3: Challenges in Histochemical Staining of Mast Cells and Strategies for Mitigation.

Challenge	Description	Mitigation Strategies	References
Nonspecific Staining (False Positives)	Other structures may take up the stain, leading to misidentification of mast cells.	- Use highly specific stains (e.g., toluidine blue with optimized pH). - Include control tissues known to lack mast cells. - Perform immunohistochemical (IHC) or immunofluorescence (IF) staining for mast cell-specific markers (e.g., tryptase, chymase).	Galli et al., 2005
Inadequate Staining (False Negatives)	Mast cells may not be properly stained, leading to underestimation.	- Optimize fixation and staining protocols. - Ensure proper tissue processing and avoid over-fixation. - Adjust dye concentration and staining duration.	Metcalfe et al., 1997
Metachromasia Variability	Staining color may differ due to variations in pH, staining time, and tissue properties.	- Standardize staining conditions (e.g., pH, incubation time). - Use freshly prepared staining solutions. - Compare with known positive controls.	Kraft & Kinet, 2007
Background Interference	High background staining may obscure mast cells.	- Improve washing steps to remove excess stain. - Use appropriate destaining techniques. - Employ counterstaining to enhance contrast.	Broome, 2012
Heterogeneity in MCs Populations	Variability in granule content, size, and staining properties makes standardization difficult.	- Use a combination of histochemical and IHC/IF markers. - Consider multiple staining techniques to account for heterogeneity.	Frossi et al., 2018
Tissue Artifacts	Processing and staining artifacts may obscure mast cells.	- Optimize tissue fixation and embedding procedures. - Avoid excessive dehydration or mechanical damage during sectioning.	Ribatti et al., 2018
Quantification Challenges	Variability in staining intensity and mast cell clustering can make counting difficult.	- Use digital image analysis for objective quantification. - Establish standardized criteria for mast cell identification. - Perform multiple counts and average results for accuracy.	Dudeck et al., 2019

distinct maturation stages, activation states, functional specializations, or microenvironmental influences within the placenta.

The enhanced staining intensity of MCs in placenta accreta cases correlated well with their increased granule content and heightened their functional activity in this experimental group.

Indeed, there is increasing evidence that mast cell morphology in the placenta is closely linked to their functional state and tissue context [40].

The quantitative analysis revealed that the total number of MCs was significantly higher in placenta accreta cases compared to normal placental tissue, suggesting a possible role in the pathogenesis of this abnormal placentation condition.

The challenges and potential problems associated with the histochemical staining techniques commonly used to detect MCs, with their suggested mitigation strategies, are shown in Table 3.

To address these challenges, researchers should standardize the staining protocols, rigorously validate staining specificity, and incorporate appropriate controls.

Moreover, combining multiple staining methods or incorporating immunohistochemical techniques is recommended to enhance

the accuracy of mast cell detection and characterization in tissues [41].

CONCLUSION

In normal placental tissue, MCs regulate angiogenesis, immune tolerance, and extracellular matrix remodeling. However, their dysregulated activity may contribute to excessive trophoblast invasion, abnormal vascularization, and chronic inflammation in PAS.

Our findings demonstrate that Toluidine Blue and the combined Alcian Blue & Safranin stains were the most effective in providing clear visualization of MCs and allowing for differentiation of subtypes, making them a valuable histological stain for MCs identification in human placental tissue.

The observed differences in MCs morphology and their increased presence in placenta accreta cases provide further insight into their potential roles in normal and pathological placentation.

Future studies utilizing immunohistochemical and molecular approaches will be essential to elucidate further the functional significance of MCs in the pathogenesis of PAS. Understanding MCs' behavior in these contexts provides insights into potential diagnostic and therapeutic targets.

Conflicts of Interests:

The authors declare that they have no financial or non-financial relationships, affiliations, or interests with any organizations or entities that could influence the content or subject matter presented in this manuscript.

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Author Contributions

All authors contributed equally to the production of this study.

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