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Original Article

Light and electron microscopic study of Haematopoiesis in the spleen of two freshwater catfishes, *Horabagrus brachysoma* and *Heteropneustes fossilis*, of Kerala

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Abstract: Haematopoiesis is the tissue-specific and developmentally regulated formation of mature blood cells from uncommitted stem cells through proliferation, commitment, and differentiation. In teleosts, blood cell formation occurs at more than one site: the head and trunk kidneys, spleen, thymus, and lymphoid tissue. In the present study, the characteristics of developing blood cells in the spleens of two indigenous freshwater catfishes in Kerala, *Horabagrus brachysoma* and *Heteropneustes fossilis*, were examined using imprint, scanning, and transmission electron microscopy. The spleen of the catfishes exhibited a few developmental stages of erythropoiesis, granulopoiesis, and lymphopoiesis, indicating the organ's role in haematopoiesis. There was a dominance of erythroid and lymphoid cells and macrophages. Lymphoid cells were usually present as aggregates in the spleen of the catfishes. The study described the ultrastructural morphology of the splenic net and the associated cells in the sections of the studied catfishes' spleen. The network of interconnected reticular cells and fibres form the meshwork for the proliferation of haematopoietic tissue and the pathway for senescent RBCs. The current work augments the available literature by providing an elaborate and complete description of the haematopoiesis in the spleen of *H. brachysoma* and *H. fossilis*.

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Introduction

The immune system of fish differs significantly from that of mammals in several aspects, including the absence of lymph nodes and bone marrow, and lymphoid tissues primarily distribute their products through circulatory pathways (Bjørgen and Koppang, 2021). The spleen is the primordial secondary lymphoid organ in fish where adaptive immune responses are generated. In teleosts, blood cell formation occurs at multiple sites, including the head and trunk kidneys, spleen, thymus, and lymphoid tissue (Mansour et al., 2025). These haematopoietic organs function either simultaneously or independently, depending on the fish species (Lange et al., 2000; Zhong et al., 2025).

Research on haematopoiesis has gained attention because an organism relies on this process, as blood cells must be continually replenished to maintain a functional immune system. A review of the literature reveals a scarcity of studies on spleen haematopoiesis, as most research has focused on the head kidney, the primary reservoir of blood cells. No substantial information is available on the cellular composition of haematopoietic organs in freshwater catfishes of Kerala. The current work expands the existing literature by examining the cellular characteristics and providing a detailed description of the spleens of two indigenous freshwater catfishes in Kerala, *Horabagrus brachysoma* and *Heteropneustes fossilis.*

Materials and Methods

Horabagrus brachysoma (Fig. 1) is an endemic catfish restricted to the rivers, backwaters, and lakes in the southern parts of India. *Heteropneustes fossilis* (Fig. 2) is a commercially important catfish, a commonly occurring species of South and Southeast Asia. Live specimens caught by local fishermen and available at landing centres and markets near the banks of the

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Figure 1. *Horabagrus brachysoma* (Günther 1864).

Figure 2. *Heteropneustes fossilis* (Bloch 1794).

Pamba River in Kerala, India (Mannar, Alappuzha district, 9.49°N, 76.33°E) were collected. Ten healthy young and adult specimens of each species, without clinical signs of disease and acclimatised to laboratory conditions, were used in the present study. The total length of *H. brachysoma* ranged from 13 to 21 cm, and body weight ranged from 34 to 60 g. The total length of *H. fossilis* ranged from 12 to 18 cm, and the weight ranged between 20 g and 42 g.

Imprint study: The fish were starved for 24 hours, sacrificed, and dissected along the ventral side of the abdomen to carefully remove the spleen. The impression technique (Ashley and Smith, 1963), in which imprints of cut portions of the organ were made on clean microslides, was employed. The air-dried imprints were fixed in methanol, stained with Wright's and Giemsa solutions (Sardar et al., 2000), and mounted in DPX. Under a light microscope (Labomed LX-400), cells were identified following the modified methodology of Fijan (2002a) and Kondera (2011) and grouped into different haematopoietic lineages. Photomicrographs were taken using Micaps 3.7 (Microview Software).

Scanning electron microscope (SEM) analysis: Small pieces of spleen were fixed in 3% glutaraldehyde prepared in Sorenson's phosphate buffer (pH 7.4, 0.1M) overnight at 4°C. Following fixation, the tissues were washed in the buffer for 30 minutes. Tissues were dehydrated in ascending grades of alcohol (30, 50, 70, 90, and 100%), with two changes each. The critical point-dried tissues were mounted on metal stubs and sputter-coated with gold. The coated tissues were examined under a scanning electron microscope (SEM, FEI Quanta 200).

Transmission electron microscope (TEM) analysis: For transmission electron microscopy, tissue samples of 1 mm^3 size were fixed in 3% glutaraldehyde in Sorenson's phosphate buffer (pH 7.4, 0.1M) for more than 24 hours at 4°C. Samples were washed with cold phosphate buffer before post-fixation in 1% osmium tetroxide (OsO4) for 2 hours. After washing with phosphate buffer, the tissues were dehydrated in

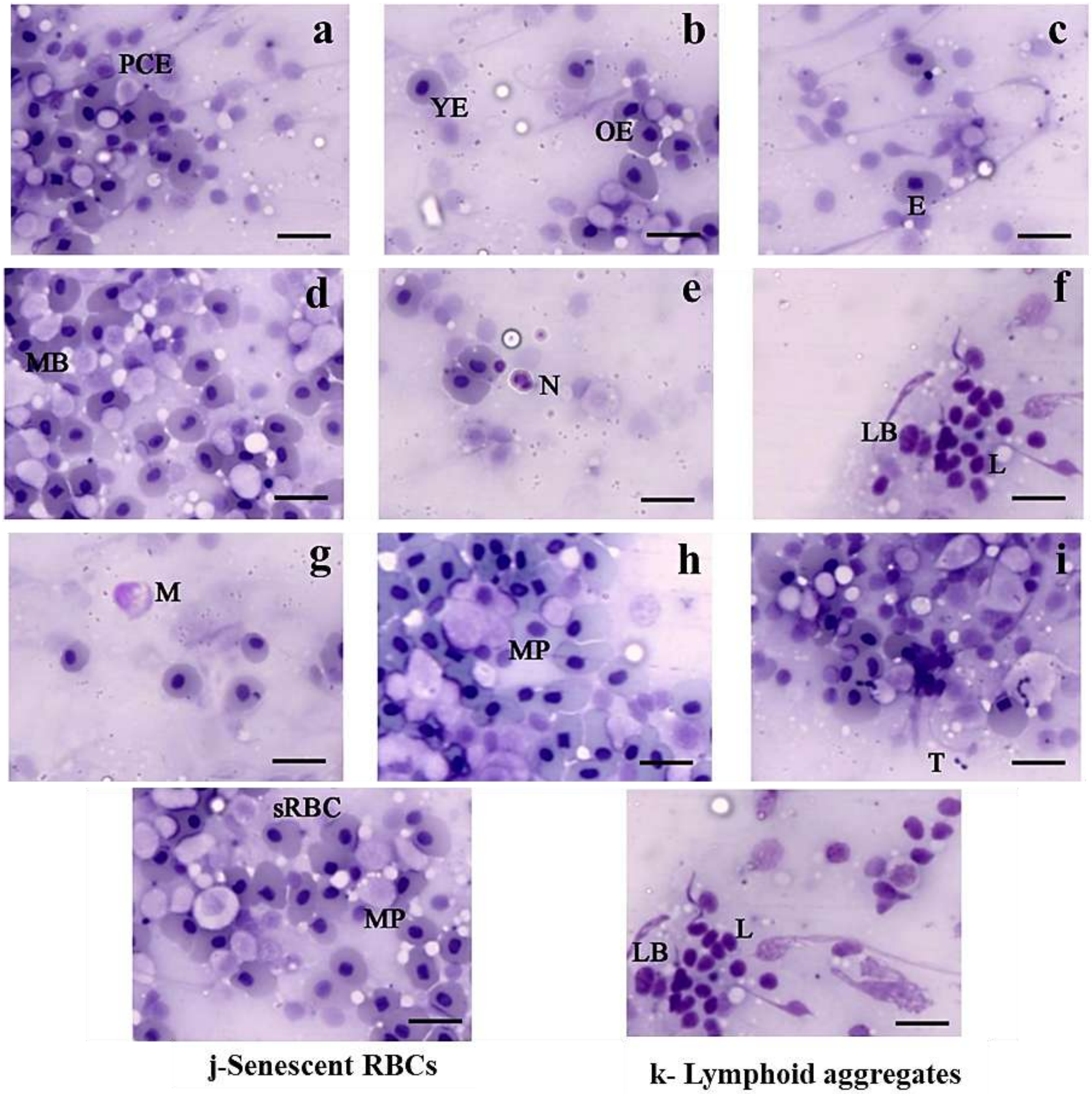

Figure 3. Developing blood cells (a-k) in the spleen of *Horabagrus brachysoma*. E: Erythrocyte; LB: Lymphoblast; L: Lymphocyte; MP: Macrophage; M: Monocyte; MB: Myeloblast; N: Neutrophil; OE: Orthochromatic Erythroblast; PCE: Polychromatophilic Erythroblast; sRBC: Senescent RBC; T: Thrombocyte; YE: Young Erythrocyte (Scale bar = 10 µm).

ascending grades of acetone. Samples were placed in propylene oxide and propylene-epoxy resin mixtures and then infiltrated with pure resin. Embedding was performed in molds containing the epoxy resin mixture according to the kit instructions (Polysciences Inc., USA), and polymerization was carried out at 60°C in an oven for three days. Ultra-thin sections (50-70 nm) cut with a diamond knife (Diatome®) were collected onto 300-mesh copper grids and stained with uranyl acetate and lead citrate. Samples were then viewed in a TEM (Hitachi H-7650) at 75-80 kV.

Results

Imprint study: Light microscopic observation of stained imprints of the spleen in *H. brachysoma* and *H. fossilis* established the presence of haematopoietic tissue. No common precursor stem cell population was identified. Staining differences for the same cells in the two fishes with Wright's and Giemsa's were sometimes noticed. The spleen of the catfishes in imprint studies exhibited a few developmental stages of erythropoiesis, granulopoiesis, and lymphopoiesis (Figs. 3, 4). In *H. fossilis,* proerythroblast, polychromatophilic erythroblast, orthochromatic

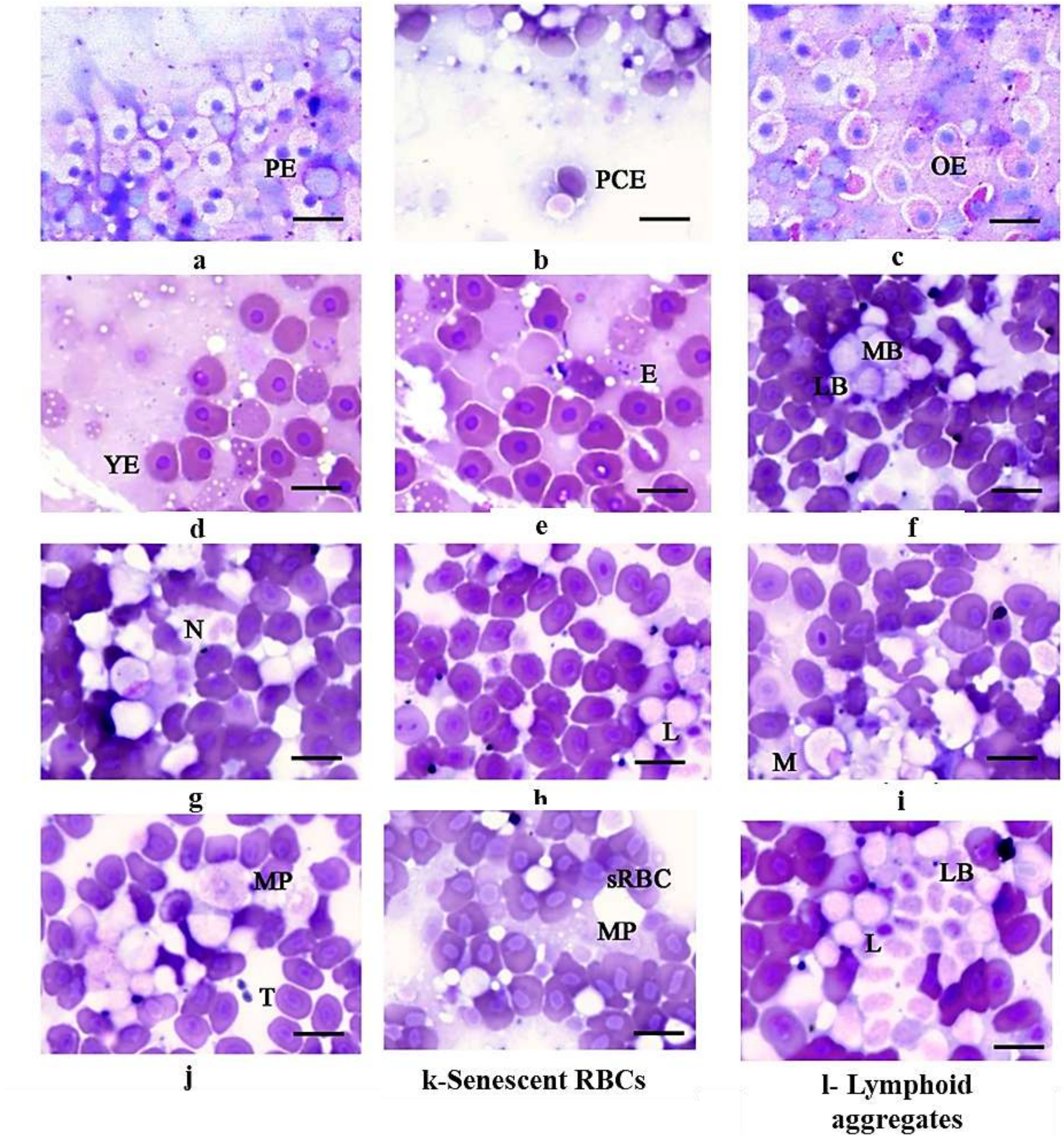

Figure 4. Developing blood cells in the spleen of *Heteropneustes fossilis* (a-i). E: Erythrocyte; LB: Lymphoblast; L: Lymphocyte; MP: Macrophage; M: Monocyte; MB: Myeloblast; N: Neutrophil; OE: Orthochromatic Erythroblast; PCE: Polychromatophilic Erythroblast; PE: Proerythroblast; sRBC: Senescent RBC; T: Thrombocyte; YE: Young Erythrocyte (Scale bar = 10 µm)

erythroblast, young and mature erythrocytes were noted as part of the erythropoietic series, while in *H. brachysoma,* only four stages, polychromatophilic erythroblast, orthochromatic erythroblast, young and mature erythrocytes were spotted. The proerythoblast was a round cell with a ring of cytoplasm around the large, diffuse chromatin. The polychromatophilic erythroblast was characterised by an oval cell shape, with a more condensed nucleus that occupied half of the cell volume. The orthochromatic erythroblast was irregularly shaped or round with an eccentric and round nucleus. The cytoplasm acquired a pale pink hue, contrasting with the bluish hue of the earlier stages. The young erythrocyte was typically round to oval, with highly condensed chromatin. The mature erythrocyte was larger than the young erythrocyte, with the most compact nucleus among the different stages of the series. The cytoplasm was stained light pink, and the nucleus was deep blue with Wright's and Giemsa's stains. Senescent erythrocytes were common

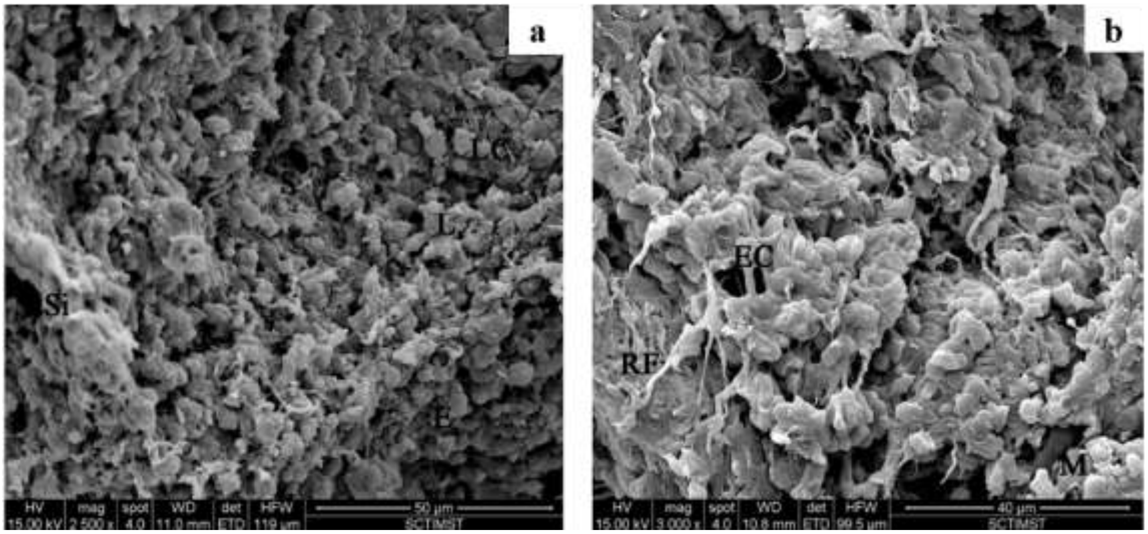

Figure 5. SEM of the spleen of *Horabagrus brachysoma*. E: Erythrocyte; EC: Erythroid cell; L: Lymphocyte; LC: Lymphoid cell; M: Macrophage; RF: Reticular Fibre; S: Sinusoid; Si: Sinus.

in spleen imprints.

In the case of granuloid lineages, only the common precursor of granulocytes, myeloblasts, and band and segmented neutrophils were recorded in the spleen imprints of the catfishes. The early precursor of granulocytes, myeloblasts, was a small round cell with a round or oval nucleus and dispersed chromatin. Neutrophils were shaped round or irregular, with a horseshoe-shaped nucleus in the band cell and a segmented nucleus in the final mature cell. The lymphopoietic series in the spleen included lymphoblasts and lymphocytes. Lymphoblasts were round or oval cells, discerned with oval, eccentric, and condensed chromatin. The cytoplasm was stained pale blue with Wright's and Giemsa stains. Mature lymphocytes varied in size, with a thin band of cytoplasm surrounding the large, centrally located, round nuclei.

Other cells in the spleen include monocytes, macrophages, and thrombocytes. The observed monocytes were round or irregularly shaped, large cells with cytoplasmic vacuoles. Nuclei were diversely shaped. Macrophages were large cells with a rough outline, sometimes with pseudopods. The almost colourless cytoplasm presented cellular debris, and the lightly-stained nucleus was situated peripherally, without a definite shape. The thrombocytes were oval, round, or elongated with compact nuclei closely resembling those in circulation. The spleen showed a predominance of erythroid and lymphoid cells, as well as macrophages. Lymphoid cells were usually presented as aggregates in the spleen of the catfish.

SEM analysis: The spleen under SEM showed numerous capillaries associated with the arterial and venous systems. The distinguished sinusoids established the predominant red pulp of the fish's spleen. The splenic net of interlocking reticular cells was the prominent feature of the fine structural analysis of the spleen. It is in this reticular meshwork and the sinuses that numerous erythroid and lymphoid cells and macrophages were observed. Mature erythrocytes were observed in various shapes and within the sinus lumen (Figs. 5, 6).

TEM analysis: Transmission electron microscopy of the spleen of *H. brachysoma* and *H. fossilis* brought to light the splenic net of reticular cells, sinusoids, constituents of the diffuse red and white pulps, and the cells of some haematopoietic series (Figs. 7, 8). The reticular cells were elongated, interconnected cells with a heterochromatic nucleus, mitochondria, and fibrils in the granular cytoplasm. The blood vessels were lined by endothelial cells, with cell processes bulging into the sinusoid lumen and characteristic desmosomes connecting adjacent cells. The cytoplasm contained RER, vesicles, and ribosomes.

As in the imprint studies, in *H. fossilis*, proerythroblast, polychromatophilic erythroblast,

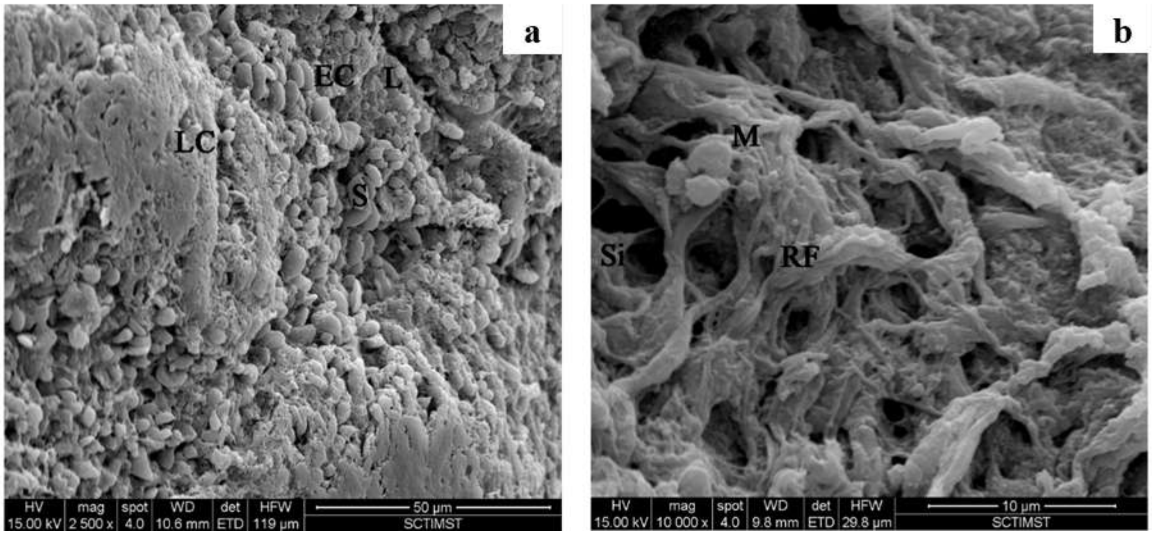

Figure 6. SEM of the spleen of *Heteropneustes fossilis*. EC: Erythroid cell; L: Lymphocyte; LC: Lymphoid cell; M: Macrophage; RF: Reticular Fibre; S: Sinusoid; Si: Sinus.

orthochromatic erythroblast, and young and mature erythrocytes were observed, whereas in *H. brachysoma*, proerythroblast was not observed. Proerythroblast was characterised by a sizeable nucleus with euchromatin and a large nucleolus. The cytoplasm contained numerous free ribosomes, vacuoles, mitochondria, and Golgi bodies. The polychromatic erythroblast had a cytoplasm mainly composed of polyribosomes and a nucleus with heterochromatin forming a ring along the edge and coarse blocks. The orthochromatic erythroblast had an almost electron-dense cytoplasm with ribosomes in rosettes and much-condensed chromatin. The young and mature erythrocytes were elongated cells with homogeneous cytoplasm, whereas the former retained some ribosomal remnants. The chromatin condensation was the highest in the mature erythrocytes. Senescent RBCs were observed in large numbers in the spleen sections, characterised by their irregular morphology.

Only myeloblasts and neutrophils of the granulopoietic series were identified in the sections. The myeloblast had basophilic cytoplasm with vesicles and ribosomes, and a large euchromatic nucleus with several nucleoli. In neutrophils, the nucleus is bilobed or kidney-shaped, and the chromatin is dense. The numerous light and dark granules present in the cytoplasm were of two types: the predominant oval type and the rod type. Organelles like RER, mitochondria, and free ribosomes were seen near the nucleus.

Lymphoid cells in *H. brachysoma* included lymphoblasts, lymphocytes, and plasma cells, whereas in *H. fossilis* only lymphocytes were identified. Lymphoblasts were characterised by a nucleus with granular chromatin and prominent nucleoli and scant cytoplasm with mitochondria, ribosomes, and vesicles. The lymphocytes were isolated from the spleen, and the cells' features included a nucleus with large heterochromatic blocks and a rim of dense cytoplasm containing free ribosomes, RER, and mitochondria. The plasma membrane occasionally presented pseudopodia. Plasma cells showed eccentric nuclei with peripheral heterochromatin blocks and the characteristic presence of well-developed dilated cisternae of the rough endoplasmic reticulum. Defined Golgi apparatus, free ribosomes, and mitochondria were also seen.

Thrombocytes of varied shapes detected in the spleen sections had heavily heterochromatic nuclei and a cytoplasm characterised by the presence of marginal microtubules and clear vesicles. Monocytes, macrophages, and melanomacrophages were distinguished in the ultra-thin sections. Monocytes were large, round cells with a jagged nucleus. The irregularly outlined cytoplasm was vacuolated and contained vesicles of varying size, a Golgi complex, RER, mitochondria, etc. Macrophages with long cytoplasmic processes were found associated with the

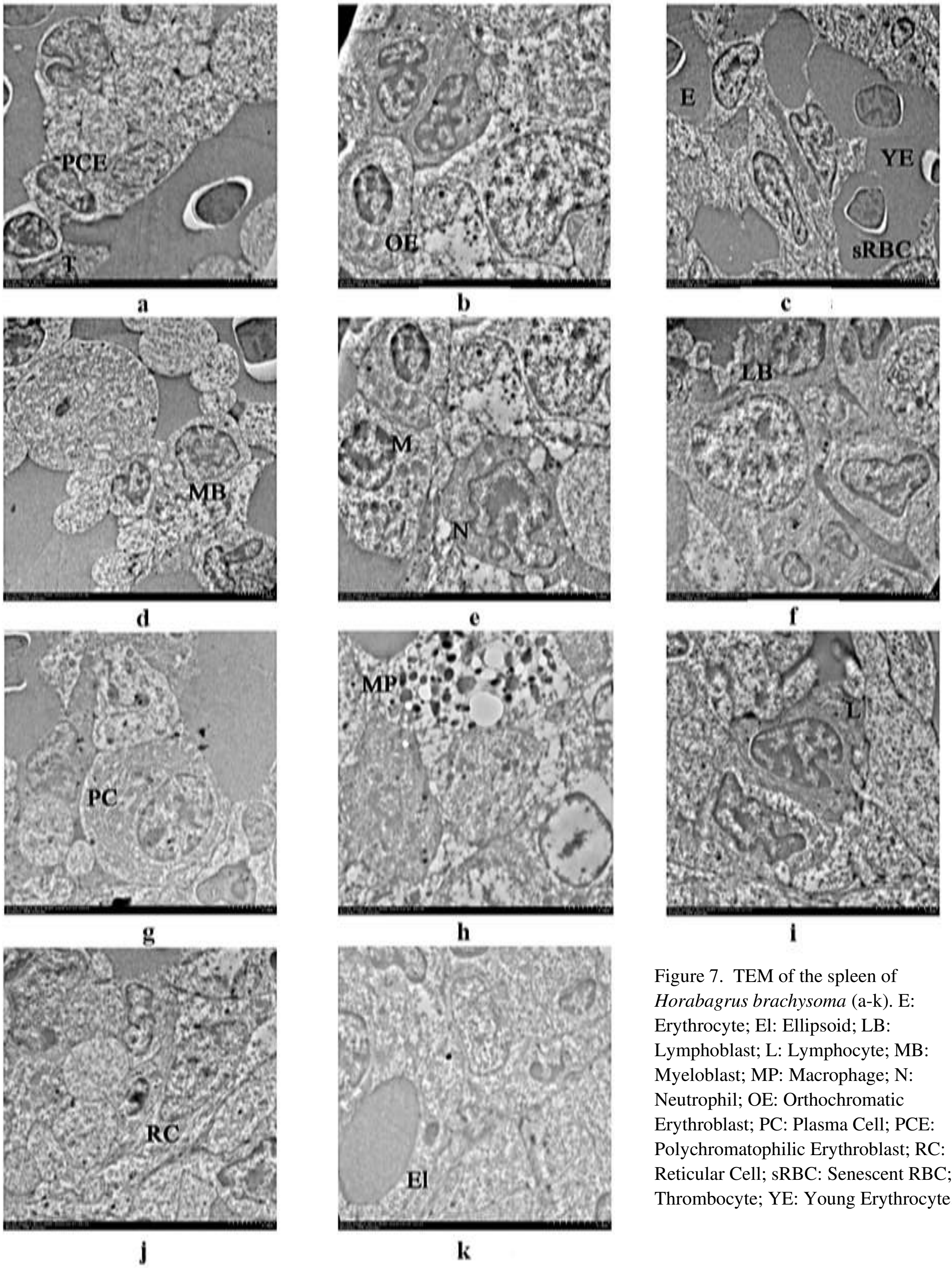

Figure 7. TEM of the spleen of *Horabagrus brachysoma* (a-k). E: Erythrocyte; El: Ellipsoid; LB: Lymphoblast; L: Lymphocyte; MB: Myeloblast; MP: Macrophage; N: Neutrophil; OE: Orthochromatic Erythroblast; PC: Plasma Cell; PCE: Polychromatophilic Erythroblast; RC: Reticular Cell; sRBC: Senescent RBC; T: Thrombocyte; YE: Young Erythrocyte.

stromal network. The cell had an eccentric nucleus with marginalised heterochromatin and noticeable nucleoli, and cytoplasm containing numerous lysosomes, phagolysosomes, heterogeneous vesicles, mitochondria, RER, the Golgi apparatus, and cellular debris. The melanomacrophages also contained pigments, such as melanin granules, observed within electron-dense vesicles. These cells occurred freely or aggregated as melanomacrophage centres (MMCs). Macrophages and melanomacrophages were observed

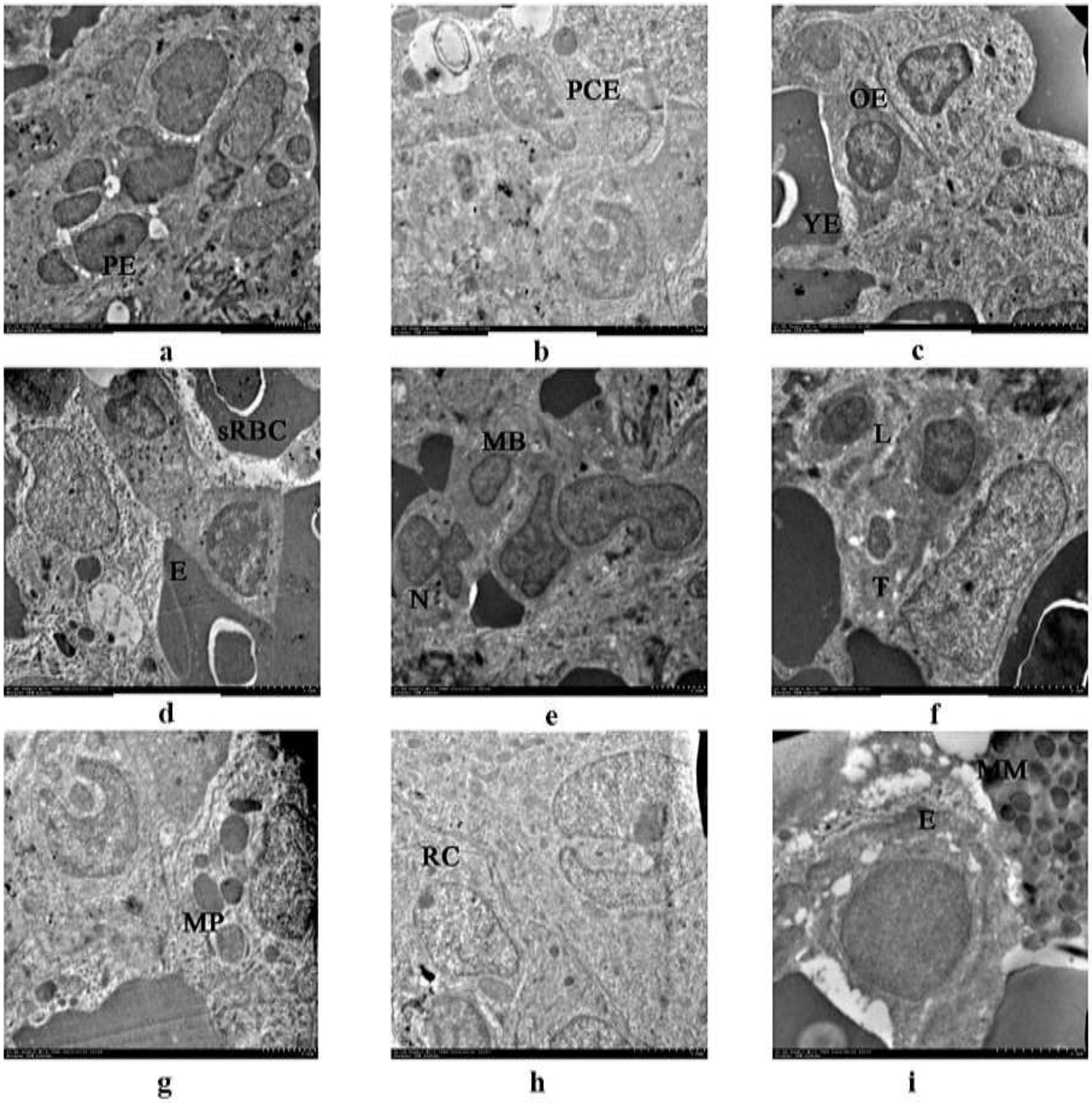

Figure 8. TEM of spleen of *Heteropneustes fossilis* (a-i). E: Erythrocyte; El: Ellipsoid; L: Lymphocyte; MB: Myeloblast; MM: Melano Macrophage; MP: Macrophage; N: Neutrophil; OE: Orthochromatic Erythroblast; PE: Prorythroblast; PCE: Polychromatophilic Erythroblast; RC: Reticular Cell; sRBC: Senescent RBC; T: Thrombocyte; YE: Young Erythrocyte.

associated with the sinusoids and ellipsoids. An ellipsoid could be recognised as a capillary lumen surrounded by endothelial and reticular cells.

Discussions

In the spleen, large sinuses and sinusoids containing RBCs constitute the red pulp, indicating the organ's role in storing and releasing blood cells (Mumford et al., 2007). Haematopoietic tissue of various lineages, supported by reticular cells in the splenic parenchyma, is involved in haematopoiesis. Deivasigamani (2002) also described haematopoietic tissue in the spleen of *Mystus gulio.* The diffuse white pulp included ellipsoid nodules, primarily lymphoid tissue around arteriole terminations, and MMCs (Wei et al., 2025). Thus, the fundamental structure of the spleen in the catfishes consisted of a vast network of blood vessels, erythrocytes, WBCs, mainly lymphocytes, and pigmented macrophages in a reticular meshwork.

To improve the knowledge of the immune organs of fishes, ultrastructural studies are indispensable. SEM analysis in the present study described the ultrastructural morphology of the sinuses and the splenic net of the catfish spleen. Arterial and venous terminals could be identified along with large sinuses. The openings between sinus-lining cells allow passage for blood cells from the reticular meshwork to the lumen. The splenic net was formed of interconnected contractile reticular cells. Deformed erythrocytes were seen passing through this meshwork, facilitating their filtration and, ultimately, degradation. These observations align with studies on the spleen in *Oreochromis niloticus* (He et al., 2021). According to Fänge and Nilsson (1985), the reticular meshwork plays a role in volume regulation of the teleost spleen, facilitating the rapid expulsion of blood cells. Erythroid and lymphoid cells also could be seen in the meshwork, evidence of haematopoiesis.

Some of the pathological studies in fish spleen employing TEM include those of Peters and Schwarzer (1985), Fishelson (2005), Wei et al. (2008), Abdelhamed et al. (2017), Lutnicka et al. (2018), etc., though the description of normal splenic architecture in these works is not elaborate. Valuable insights into the structure of the spleen and the mechanism of hematopoietic development in yellowfin tuna were presented in the recent study by Guan et al. (2025). The major feature of the stroma of the spleen, under TEM, was the network of interconnected reticular cells and fibers forming the splenic net. These cells form the meshwork for the proliferation of haematopoietic tissue and the pathway for senescent RBCs. Quesenberry et al. (1994) proposed that the stroma influences haematopoiesis. Lymphocytes and macrophages were closely associated with the splenic net, indicating their role in antigen presentation. Similar morphology under TEM can be observed in *O. niloticus* (He et al., 2021). In the spleen of *Mustelus schmitti,* Galindez and Aggio (1998) identified two types of reticular cells, light and dark, though their functionality is not clearly demarcated. In the spleen of *Tilapia mossambica,* no such divisions of reticular cells are noted (Sundaresan, 2014). The irregular endothelial cells of the sinusoidal lining function as a barrier between blood and haematopoietic tissue, though allowing active transport of water and nutrients. A vital part of the white pulp, the ellipsoids, could be distinguished as a capillary lumen with reticular fibrous covering. These structures formed a close association with the phagocytic cells. The fine morphology of the ellipsoids was comparable to that of *O. niloticus* (He et al., 2021).

In the haematopoietic organs, the common precursor, the haematopoietic stem cell, gives rise to a haematopoietic progenitor cell that creates daughter cells of all blood cell lines (Kondera, 2019). In a comparative study on haematopoiesis in air-breathing fishes, Vergis (1992) identified the stem cell found in the head kidney and spleen as a haemoblast. No such stem cells or early blast cells were identified in the present study, consistent with the report by Fijan (2002b) on channel catfish. In the present work, the spleen of the catfish, even with only a few developmental stages of erythropoiesis and granulopoiesis, showed a dominance of erythroid and lymphoid cells. It was noted that the different cell types intermingled regardless of lineage.

The number of erythropoietic stages and their terminology vary across many fish, but researchers agree that the series is the most frequently observed, with the highest number of developmental stages in the head kidney and spleen (Liu et al., 2017; Zhong and Yan, 2024). The erythroid lineage cells in the spleen of *H. brachysoma* were mainly found together in ultra-thin sections, representing the red pulp. The detection of four to five stages of the erythroid lineages in the catfish indicated the part played by the spleen in erythropoiesis and blood retention. Immature erythrocytes are seen in the spleen of *T. mossambica* (Sundaresan, 2014) and *Epinephelus tauvina* (Patra, 2006) spleen, though they are not further characterised. In the spleen of *M. schmitti,* Galindez and Aggio (1998) identified different cells of the erythropoietic series, suggesting a close relationship between erythroblasts and melanomacrophages. Throughout the erythropoiesis in the catfish spleen, there was a gradual increase in the condensation of chromatin, similar to the observations by Gangopadhyay (2005) and Gangopadhyay and Homechaudhuri (2011). With each stage, the cytoplasm changes from blue to light pink due to the gradual accumulation of haemoglobin

(Liu et al., 2017). The ratio of cytoplasm to nucleus also increased, though the cell size fluctuated. This is in accordance with the results of Kondera (2014) in the head kidney of *Squalius cephalus*, whereas El-Saydah et al. (2010) in the head kidney of *O. niloticus* reported a decrease in cellular size during maturation. In the erythropoietic series, there is replacement of cytoplasmic organelles during maturation, as noted in the study of Santos et al. (2011). Ribosomes are active in the early stages of the series, with progressive degradation toward the mature stage. The organelle may be involved in ongoing haemoglobin synthesis in erythroid cells until saturation (Majeti et al., 2007; El-Saydah et al., 2010). Large numbers of irregularly shaped senescent RBCs in the spleen, as well as imprints and ultra-thin sections, indicated the organ's active role in the degradation and clearance of effete blood cells (Grizzle and Rogers, 1976).

The common precursor of granulocytes, myeloblast, was detected in spleen imprints of the catfish. This highlights the organ's role, albeit less active, in granulopoiesis, even though subsequent developmental stages may not be reported. In the spleen imprints of the studied fishes, apart from myeloblasts, only band and segmented neutrophils could be identified. Basophilic and eosinophilic series were not observed. The ultrastructural pattern of the identified neutrophils follows the description in the study by Mokhtar and Abd-Elhafez (2021). The numerous light and dark granules included the predominant oval and rod types. The presence of cytoplasmic granules increased in the mature neutrophils.

In the spleen of the studied catfishes, the lymphopoietic series included lymphoblasts and lymphocytes. This observation is consistent with Vergis's (1992) comparative studies on air-breathing fishes. In some TEM studies (Galindez and Aggio, 1998; Sundaresan, 2014), no precursor cells were recognized in the fish spleen. Patra (2006) has marked lymphoblasts in the *E. tauvina* spleen. In the spleen, the blast and mature cells were commonly presented as lymphoid aggregates. These aggregates might correspond to the white pulp portion. From the abundance of lymphocytes in the spleen, it can be stipulated that the organ is active in lymphopoiesis in the studied catfishes. The occurrence of plasma cells accounts for the organ's direct involvement in immune response also. The presence of plasma cells established the organ as a site for B-cell proliferation.

Other cells identified in the spleen include monocytes, macrophages, and thrombocytes. Monocytoid cells were reported in the fish head kidney by Nayak (1982) and Liu et al. (2017). Typical macrophages and melanomacrophages were located near the sinusoids and associated with the splenic net in our study, consistent with the results of Zhong et al. (2025), further underscoring their role in immunity. Macrophages were more abundant than monocytes in the organ. In the current work, macrophages were the largest cells identified in the imprints. Their cytoplasm was easily recognisable with a rough outline, presence of cellular debris and vacuoles, sometimes with parts of senescent RBCs. Macrophages were identified in the haematopoietic studies by Fijan (2002b), El-Saydah et al. (2010), Liu et al. (2017), etc.

The thrombocytes found in the organ were morphologically similar to those in peripheral blood smears, but they also had clear vacuoles and marginal microtubules. Similar to our study, only mature thrombocytes were singled out in the hematopoietic tissue of *Rasbora daniconius* (Nayak, 1982), some air-breathing fishes (Vergis, 1992), and *S. cephalus* (Kondera, 2014), while thrombopoietic stages were noted in the head kidney of *O. niloticus* (El-Saydah et al., 2010) and *Cyprinus carpio* (Suljevic et al., 2016). The information on the morphology and nomenclature of thrombocyte precursors is yet to be habituated. The present study identified no thrombocyte precursors in the spleen of the catfish studied.

Conclusion

The present study proves that the spleen, a major immune organ in teleosts, has an active role in haematopoiesis. The study successfully employed electron microscopic analyses to gain a finer understanding of the spleen in freshwater catfishes. The spleens of *H. brachysoma* and *H. fossilis* showed dominance of erythroid, lymphoid, and macrophage cells. Lymphoid cells were usually presented as aggregates in the spleen of the studied catfishes. The significance of all these cells of haematopoiesis, involved in immune response, warrants much detailed study on the haematopoiesis in the organ.

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