

Descriptive characteristics of haemopoietic cell lineages in a facultative air breathing fish *Clarias batrachus* (L.)

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Abstract: Haemopoietic tissues of the head kidney of *Clarias batrachus* were studied for morphometric analysis and ultrastructural descriptions of the erythropoietic and leucopoietic cell populations. Eight successive steps of developing erythrocytes from small lymphoid haemoblasts (SLHs) to mature erythrocytes (MEs) were distinguished. Progressive loss of RNA and synthesis of haemoglobin during haemopoiesis were coupled with denser nucleus and changes in cellular shapes. Transmission electron microscopy aided in clear distinction between cell lineages in the development of erythrocytes, granulocytes, and lymphocytes. Ultrastructural analysis of the pronephric stromal cells forming adherent multilayer of haemopoietic microenvironment was explicit in describing their ability to promote haemopoiesis in fish. Barrier cells observed within the haemopoietic tissue indicate their functional significance in the haematopoietic microenvironment.

Key words: Haemopoietic tissue, head kidney, cat fish, ultrastructure

Introduction

Vertebrate blood cells originate from multipotent or uncommitted stem cells, which are localised in lymphomyeloid or haemopoietic tissues. In teleosts, the anterior part of the kidney (head kidney or pronephros) is transformed into complex lymphomyeloid or haemopoietic tissues. The lymphomyeloid tissues in fish consist of a mixture of lymphoid and myeloid compartments. However, they are regarded as being less specialised in their structure and function than the corresponding mammalian tissues. In fish, analysis of cell associations in the haemopoietic tissues is of great importance in the study of haemopoiesis and the formation of haemopoietic microenvironments.

There are a few sets of data on the blood cell lineages in haemopoietic organs of teleosts (Wlasow and Dabrowska, 1989; Quentel and Obach, 1992; Maiti et al., 2000; Fijan, 2002; Zapata et al., 2006). However, such information is lacking for most of the Indian fishes. Investigations on the ultrastructure of haemopoietic tissues and cells have been reported in various fish species but most of these deal with limited cell types (Weinreb, 1963; Ferguson, 1976; Morrow and Pulsford, 1980; Blaxhall, 1983; Fujimaki and Isoda, 1990; Qionglin and Quanzhang, 1994; Tripathi et al., 2004; Fishelson, 2006). The freshwater catfish, *Clarias batrachus* Linnaeus 1758, a food fish and an inhabitant of shallow ponds and lakes, is a facultative air breathing fish, the population of

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which is in steady decline due to eutrophication and consequent oxygen stress. Further, acidified aquatic systems lead to diseases which, when at levels beyond the threshold, contribute to increased mortality of the fish population. Therefore, to understand the fish's response to such stressors, the present study is directed towards the characterisation of haemopoietic cells in the lymphomyeloid tissues of the pronephric kidneys, both by light and electron microscope studies, so that immunopathological investigations can be carried out for diagnostic purposes.

Materials and methods

Different stages (fry, fingerling, and adult) of *C. batrachus* were obtained from Kulia fish farm, Kalyani, West Bengal, and acclimatised in the laboratory for 3 weeks. Fish were fed daily with commercial pelleted feed. Water temperature (23-24 °C), dissolved oxygen (4.5-5.5 mg L⁻¹) and dissolved free carbon dioxide (trace), pH (7.2-7.6), alkalinity (120-140 mg L⁻¹), and hardness (230-240 mg L⁻¹) were monitored during the process of acclimatisation.

For haemopoietic study, the imprint or impression technique was employed following Mahajan and Dheer (1980a). A small piece from the head kidney (pronephric kidney) was carefully dissected out and a tissue imprint was made on clean glass slides. The preparation was air dried and stained with Graham Knoll's benzidine method (Forteza Bover, 1964), followed by counterstaining with Giemsa (Mahajan and Dheer, 1979).

The cells from haemopoietic organs were measured by ocular micrometer (LM magnification 10 × 100). Area of the circular cells were calculated from πr^2 , where r = half of the cell diameter, and that of the elliptical cells from πab , where a = half of major axis and b = half of minor axis.

Tissues from the head kidney of 18 days, 30 days, 60 days, and of the adult fishes were dissected out and fixed in 4% glutaraldehyde solution (Karnovsky, 1965) in phosphate buffer (pH 7.2) and kept at 4 °C for 6 h, and this was followed by washing in double distilled water. The tissues were postfixed in 2% aqueous OsO₄ solution for 2 h at room temperature. After repeated washes in water, the tissues were dehydrated in upper graded alcoholic series and embedded in araldite

CY212. Sections (60-70 nm) were then cut with an ultramicrotome (Reichert ultracut E) mounted on a copper grid contrasted with uranyl acetate and lead citrate. Sections were observed in a Phillips CM-10 transmission electron microscope at AIIMS, New Delhi, at accelerating voltage of 80 kV.

Results

A large mass of pronephric kidney as haemopoietic tissue indicated its role as the principal haemopoietic system in *C. batrachus*. In the present study (Table), the first stage of the erythrocyte lineage was the proerythroblasts or small lymphoid haemoblasts (SLHs). SLHs were round cells with an extensively basophilic cytoplasm and a large, central, circular nucleus. The proerythroblasts progressively lost RNA from the cytoplasm, and synthesised haemoglobin; the nucleus got denser and the cell outline changed from circular to elliptical. The 8 successive stages of developing erythrocytes in *C. batrachus* were: i) proerythroblast or small lymphoid haemoblast (SLH), ii) basophilic erythroblast (BE), iii) polychromatophilic erythroblast (PE), iv) acidophilic erythroblast (AE), v) young reticulocyte (YR), vi) mature reticulocyte (MR), vii) young erythrocyte (YE), viii) mature erythrocytes (ME). The leucocytic cells observed in haemopoietic tissues were lymphocyte (LYM), neutrophil (NEU), and macrophages (MACR). Ultrastructural analysis of the pronephric stromal cells of *C. batrachus* of 18 days, 30 days, and 60 days of the adult were carried out.

Transmission electron microscopy revealed the fine structure of a large number of developing cells of the erythrocytic, granulocytic, and lymphocytic lineages in the head kidney. However, no structural variations in the pronephric cell population in fishes of different ages were observed. Different haemopoietic cells had been identified from the following features.

Young reticulocyte (YR) was oval to elliptical with a large oval or round nucleus and irregular shaped nucleolus. Mature reticulocyte (MR) showed an ellipsoidal profile with slight tapering towards the end of the long axis. The nucleus was diminished in volume compared to YR. A bundle of microtubules was located near the 2 tapering ends, forming a marginal band (Figure 1).

Table. Characteristics of haemopoietic cells in *Clarias batrachus*.

Cell Type	Shape		Area (μm^2)		N-C ratio	Staining ability		Percentage distribution
	Cell	Nucleus	Cell	Nucleus		Cytoplasm	Nucleus	
SLH	Round	Round	19.63 \pm 2.12	12.56 \pm 1.76	1:1.56	Purple	Deep purple	22.91 \pm 1.97
BE	Round	Round	73.63 \pm 3.72	35.06 \pm 2.55	1:2.10	Blue	purple	9 \pm 1.15
PE	Round	Round	132.67 \pm 2.24	63.59 \pm 12.84	1:2.09	Light purple	Purple	8.53 \pm 1.76
AE	Round	Round	107.02 \pm 5.21	38.47 \pm 2.55	1:2.78	Pink	Purple	3.49 \pm 0.65
YR	Round to oval	Round	69.55 \pm 4.30	12.56 \pm 1.58	1:5.54	Brown	Purple	7.15 \pm 0.39
MR	Round to oval	Round	69.61 \pm 14.0	7.07 \pm 3.14	1:9.85	Brown	Purple	13.20 \pm 2.85
YE	Round to oval	Round	73.53 \pm 4.08	7.07 \pm 1.58	1:10.40	Brown	Purple	9.13 \pm 1.07
ME	Elliptical	Round	61.82 \pm 8.09	12.56 \pm 1.58	1:4.92	Brown	Purple	7.87 \pm 1.34
LYM	Round	Round	10.282 \pm 2.32	6.09 \pm 1.69	1:1.688	Pinkish-white	Dark pink	12.85 \pm 1.67
NEU	Round	Round to lobed	26.10 \pm 5.589	12.00 \pm 5.22	1: 2.17	Light pink	Dark pink	17.16 \pm 2.58
MACR	Round	Round	72.53 \pm 8.172	11.77 \pm 5.17	1: 6.16	Pale blue-grey	Pink	2.15 \pm 1.38

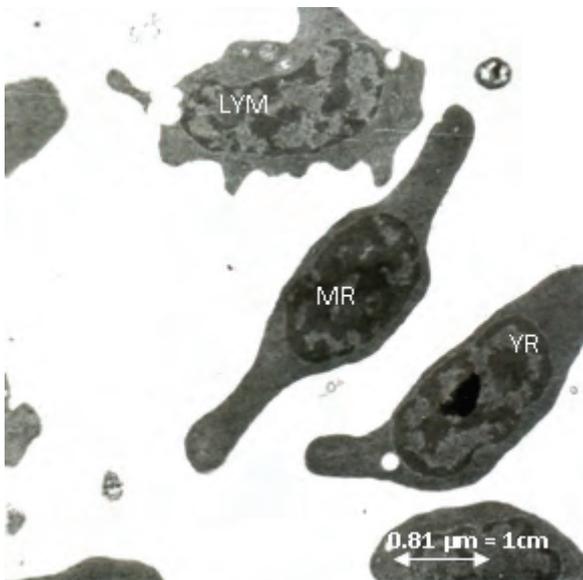


Figure 1. Transmission electron micrograph of young reticulocyte (YR), mature reticulocyte (MR) with large nucleus, and lymphocyte (LYM) with electron dense nucleus occupying most area of the cell from head kidney of 18-day-old *Clarias batrachus* (Scale bar = 0.81 μm).

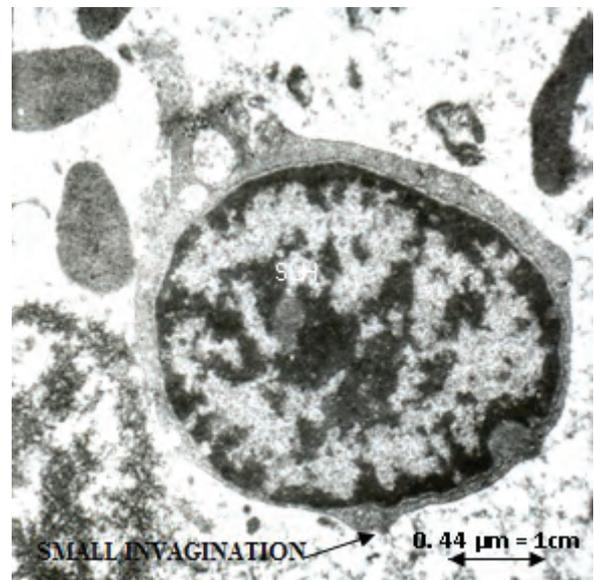


Figure 2. Transmission electron micrograph of small lymphoid haemoblast (SLH) with characteristic large nucleus and rim of cytoplasm from head kidney of 60-day-old *Clarias batrachus* (Scale bar = 0.44 μm).

Small lymphoid haemoblast (SLH) type of cells (Figure 2) were round with a large nucleus and irregular shaped nucleolus. Nucleoplasm was mostly homogeneous except for small patches of electron opaque material and chromatin along the inner surface of the nuclear envelope. The plasma

membrane showed small invagination due to micropinocytic activity of the cell.

Lymphocyte (LYM) was the smallest of leukocytes having a large nucleus surrounded by a thin rim of cytoplasm. Plasma membrane was plicate and occasionally having small pseudopodia (Figure 1).

Electron-dense chromatin was present. The nucleus was often indented or clefted. Small cytoplasmic vesicles were present (Figure 3).

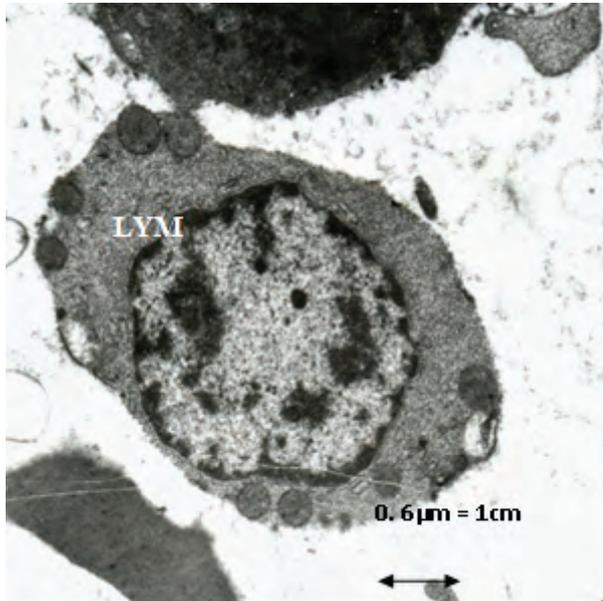


Figure 3. Transmission electron micrograph of lymphocyte (LYM) from head kidney of 18-day-old *Clarias batrachus* (Scale bar = $0.46 \mu\text{m} \times 13,800$).

Neutrophils (NEU) (Figure 4) were the most abundant leukocytes with irregular and occasionally bilobed nucleus. The nuclear chromatin material was dense and patchy in distribution. The cytoplasm was characterised by the presence of numerous granules scattered in cytoplasm. Two types of granules were identified based on their shape and size. Type I granules were membrane bound, oval, large, and predominant in the cytoplasm. Type II granules were rod-like. Neutrophils containing both types of granules were fewer in number than neutrophils, having only Type I granules. Both types of granules were observed in differentiated cells.

Monocytes (MONO) were irregular in shape with a large nucleus (Figure 5). The nucleus was relatively electron lucent apart from a narrow peripheral band of chromatin and apparent large nucleolus. Cytoplasm contained vesicles of varying size and electron density.

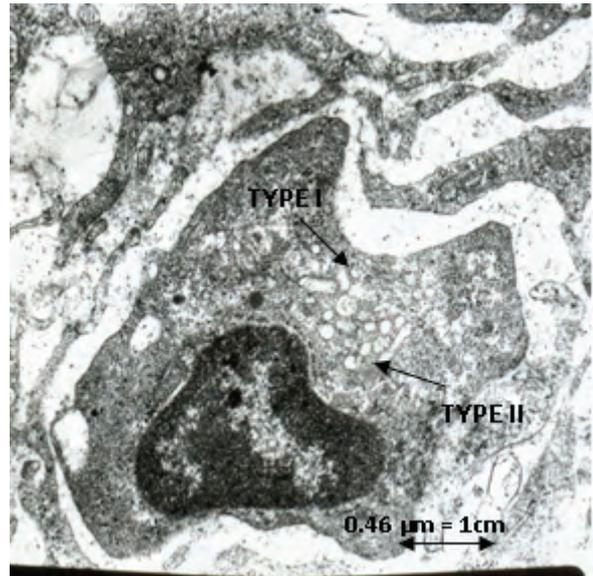


Figure 4. Transmission electron micrograph of neutrophil (NEU) with eccentric nucleus and characteristic Type I and Type II granules from head kidney of 60-day-old *Clarias batrachus* (Scale bar = $0.46 \mu\text{m}$).

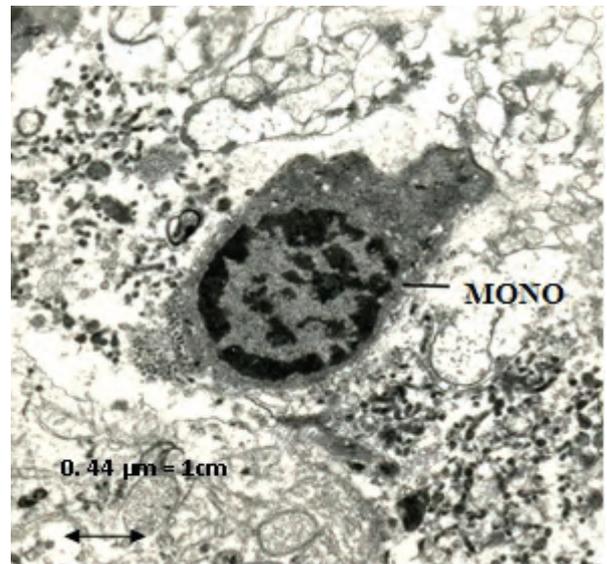


Figure 5. Transmission electron micrograph of monocyte (MONO) from head kidney of mature *Clarias batrachus* (Scale bar = $0.81 \mu\text{m}$).

Spherical aggregate of melano-macrophages (MM) (Figures 6 and 7) with pigments containing vesicles were found throughout the haemopoietic kidney.

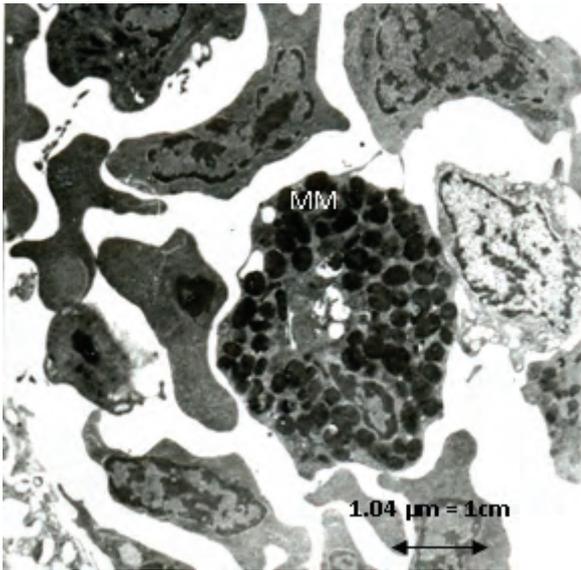


Figure 6. Transmission electron micrograph of melanomacrophage (MM) containing dense melanin granules from head kidney of 18-day-old *Clarias batrachus* (Scale bar = 1.04 μm).

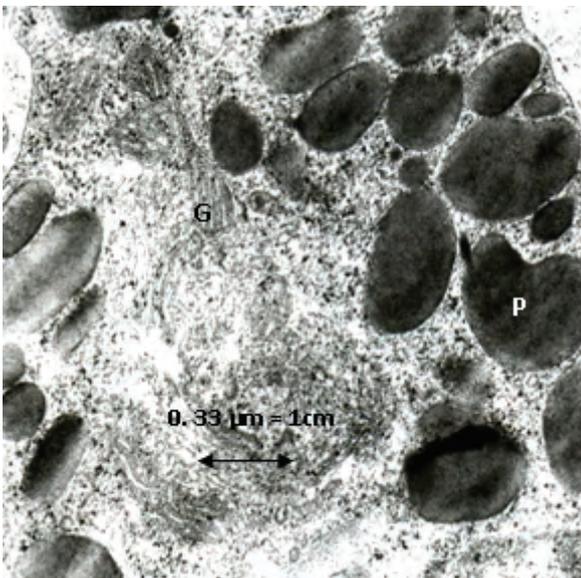


Figure 7. Transmission electron micrograph of pigment granules (P) and Golgi bodies (G) from melanomacrophage cell of head kidney of mature *Clarias batrachus* (Scale bar = 0.33 μm).

Mature erythrocytes (ME) were elliptical with an irregular lobulated nucleus, further decreased in volume compared to MR. The chromatin was highly

condensed into large electron opaque blocks. The nucleolus was no longer identifiable. Thrombocytes (THR) were elongated, fusiform cell with an indentation in the plasma membrane. Numerous coated vesicles were present in the cytoplasm (Figure 8).

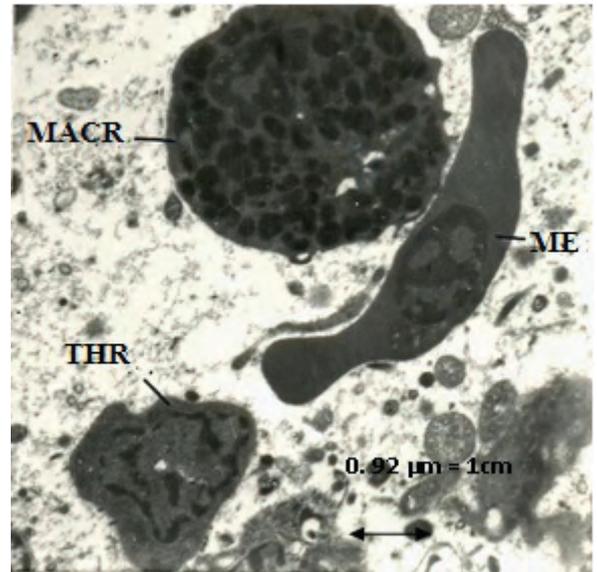


Figure 8. Transmission electron micrograph of macrophage (MACR), mature erythrocyte (ME), and thrombocyte (THR) with canalicular and granular structure from head kidney of 18-day-old *Clarias batrachus* (Scale bar = 0.8 μm).

Macrophages (MACR) were irregular in shape and characterised by indented nucleus (Figure 8). The plasma membrane showed long processes of pseudopodia. Actively phagocytosing macrophages (Figure 9), very few in number, were seen within the head kidney. The cell cytoplasm was replete with lysosomes, mitochondria, pigments, and other particles.

Myoblast (MYO) (Figure 10) was round with small nuclei. Small granules were present in cytoplasm. Barrier cells (BC) were few in number with electron-dense, elongated, and branched appearance (Figures 10 and 11). Numerous ribosomes, well-developed secretory organelles, and electron-lucent vesicles characterised the cells. Large granules and microfilaments were also present.

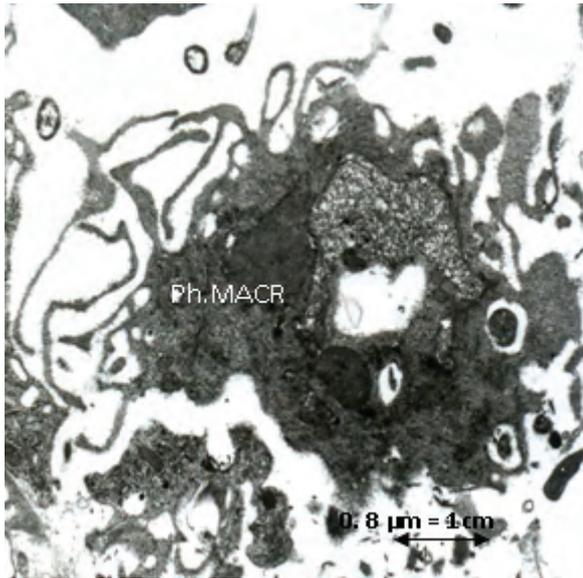


Figure 9. Transmission electron micrograph of phagocytic macrophage (Ph. MACR) with cytoplasmic processes and engulfed material from head kidney of 18-day-old *Clarias batrachus* (Scale bar = 0.8 μm).

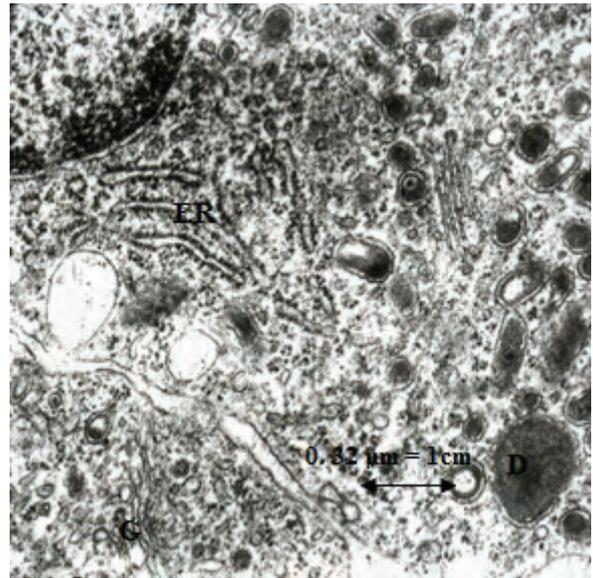


Figure 11. Transmission electron micrograph of barrier cells with Golgi bodies (G), membrane bound droplets (D), and endoplasmic reticulum (ER) from head kidney of mature *Clarias batrachus* (Scale bar = 0.32 μm).



Figure 10. Transmission electron micrograph of barrier cells (BC) and myoblast (MYO) from head kidney of 18-day-old *Clarias batrachus* (Scale bar = 1.4 μm).

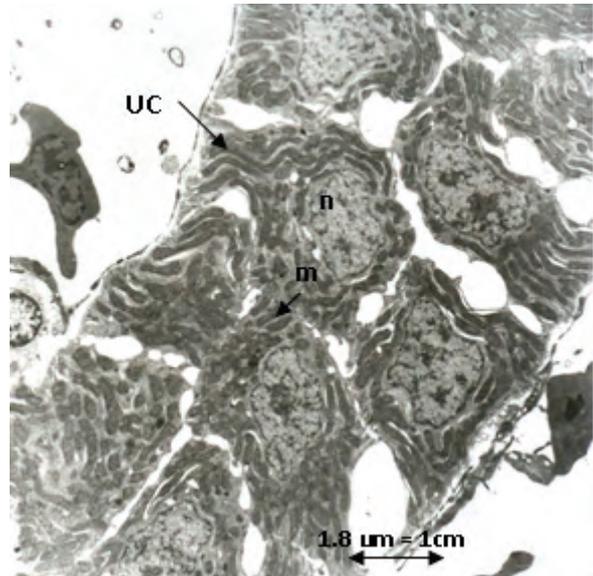


Figure 12. Transmission electron micrograph of unidentified cells (UC) with centrally located large nucleus (n) and cytoplasm filled with mitochondria (m) (Scale bar = 1.8 μm).

Few unidentified cells with large number of mitochondria (Figure 12) were also observed. Cells were either isolated or formed of syncytial networks. Position-wise, they were found lining the blood

sinusoids of the renal haemopoietic tissue and closely associated with erythropoietic and plasmacytopoietic foci.

Discussion

In the present study, apart from the characteristic differences in the developmental stages of the erythrocytic lineage, a progressive increase in the N-C (nucleus to cytoplasm) ratio from SLH stage to ME stage (Figure 13) is evident. This change is due

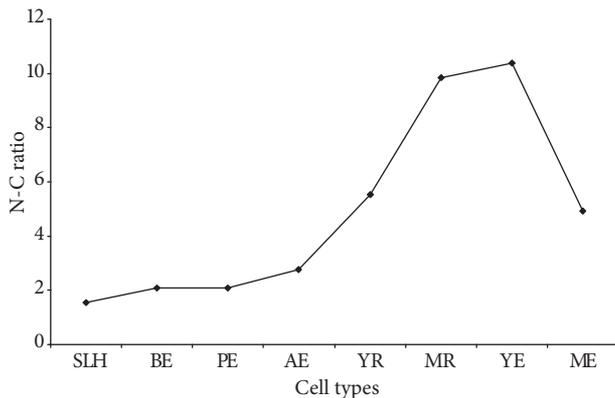


Figure 13. N-C ratio of erythropoietic cell lineage in head kidney of *Clarias batrachus*.

to condensation of nucleus and gradual increase in cytoplasm as DNA synthesis is known to occur only in the erythroblast stages (BE, PE, and AE) but not during reticulocytes formation (Mahajan and Dheer, 1980b). The transformation from initial basophilic cytoplasm in BE to acidophilic one in AE is significant because an iron-porphyrin compound in haemoglobin is formed by incorporation of iron into erythroblast in acidic conditions (Granick, 1953; Maiti et al., 2000). Development of acidophilic cytoplasm, therefore, initiates the formation of haemoglobin streaks in YR and quantitative increase of haemoglobin mass in MR. AE seems to be an intermediate transient stage, giving rise to reticulocytes with the incorporation of iron. Later the mature erythrocytes become elliptical due to the formation of an equatorially oriented marginal band of microtubules (Sekhon and Beams, 1969). Shrinkage of cytoplasm and the subsequent decrease in cell area might be caused by disintegration and disappearance of cell organelles like the mitochondria, the Golgi complex, and the centrioles during the process of maturation. In *C. batrachus*, the high percentage of stored SLH than other cell types is significant since blood oxygen carrying capacity can be increased by release of stored cells (Murad et al., 1990). This

might be an adaptive strategy for sudden hypoxic conditions in shallow habitats.

In fish, for understanding haemopoiesis, analysis of cell associations that form haemopoietic microenvironment is necessary. Previous studies (Diago et al., 1993; Flano et al., 1998) have shown that haemopoiesis is dependent on the establishment of an adherent layer of stromal cells, which provide appropriate environments to promote the survival, self-renewal, proliferation, and differentiation of the stem cells. In mammals, the study of the function of stromal cells supporting haemopoiesis has involved the use of clones or lines of adherent cells from long-term cultures (Henderson et al., 1990; Novotny et al., 1990; Pessina et al., 1992). Stromal cells differ in their ability to promote expansion and maturation of haemopoietic precursor cells, in the expression of cell surface antigens, and in the production of the constitutive growth factor. In fish, some stromal cell lines have also been developed (Cheng et al., 1993; Fryer and Lannan, 1994), but their ability to support haemopoiesis has not been studied as yet. In this study, the ultrastructural analysis of the pronephric stromal cell composition has been done.

In *C. batrachus*, a network of reticulo-endothelial cells supports the haemopoietic parenchyma in the intertubular spaces of the pronephros. Thin-walled blood vessels communicate with the haemopoietic pulp, which comprises developing and mature erythrocytes and granulocytes. Spherical aggregates of melano-macrophages are found throughout the haemopoietic kidney. These aggregates are bounded by a thin fibrous membrane and surrounded by a white pulp devoid of the erythrocytic series of cells. The aggregation of macrophages especially within and adjacent to the melanin centres is important since such aggregations of melano-macrophages and lymphoid elements have significance for the antibody production in immune response.

The maturational changes of the ultrastructure in the series of erythroid cells depict the similar characteristics in having transformation from a spherical to flattened shape, shrinking of the nucleus, condensation of the chromatin, reduction in the number of ribosomes and other organelles, and increase in the electron-opacity of the cytoplasmic matrix due to accumulation of haemoglobin. Such

changes closely parallel the features seen in the maturation of erythroid cells in other fish and vertebrates that have been studied (Sekhon and Beams, 1969; Sekhon and Maxwell, 1970; Barrett and Scheinberg, 1972).

In teleosts, granulocytes originate together with the erythrocytes in the renal lymphomyeloid tissue. In a number of animals, including fish species, neutrophils have been reported to possess several kinds of granules (Watanabe et al., 1974; Suzuki, 1986; Hine, 1992). In *C. batrachus* 2 types of neutrophil granules are identified. Type I granules are seen in all neutrophils, and both Type I and Type II granules are observed in the mature neutrophils only. Based on the ultrastructure earlier described, it is opined that Type I granules are azurophil ones whereas Type II granules are specific types. In lymphocytes of humans, scattered and clustered dense bodies and their distributional patterns related to other organelles determine their classification as B- lymphocytes or T-lymphocytes (Watanabe et al., 1974). In this study, such scattered and clustered dense bodies are also distinct in lymphocytes which, however, could not be classified into any subpopulation based on the distributional pattern. Similar observation was made by Fujimaki and Isoda (1990) on fish lymphocytes. The monocytes are characterised by an electrolucent pleomorphic nucleus, abundant cytoplasm, and many mitochondria as well as rough endoplasmic reticular and well-developed filopodia. It is, therefore, reasonable to treat the monocytes as an independent cell group in fish (Morrow and Pulsford, 1980; Suzuki, 1986; Bielek et al., 1999).

The nucleated thrombocytes can be distinguished from other cells in having extensive surface connected canicular system of the cytoplasm, as seen in the carp thrombocytes (Daimon et al., 1979) and are analogous to mammalian platelets (Behne, 1968).

As is known, macrophages accumulate several kinds of pigments of which predominant ones are melanin, lipofuscin, and haemosiderin. Melanin is produced by melanocytes and is contained within spherical to ovoid melanosomes that are about 1-4 μm in diameter (Sealy et al., 1980). Lipofuscin is derived from oxidation of polyunsaturated fatty acids. Haemosiderin is a ferric ion rich by-product of haemoglobin breakdown (Grove, 1968). In primitive

fishes such as salmonids, pigmented macrophages are well dispersed in the anterior kidney, spleen, liver, and elsewhere (Agius, 1980, 1981). In contrast, higher fishes have discrete aggregation of pigmented macrophages (macrophage centre) in the corresponding soft tissue. In *C. batrachus*, distinct melano-macrophage centres are seen in the head kidney and are suggestive of analogy with the germinal centres of higher vertebrates. Such a macrophage centre with high level of pigmentation in fish is significant because poikilotherms need high levels of unsaturated fats in their tissues to maintain the membrane fluidity essential for normal metabolic processes even at low temperature. These unsaturated fats are particularly prone to peroxidation with the possible formation of pigments that concentrate in macrophage centres. Melanomacrophage centres act as focal depositories for resistant intracellular bacteria. In addition to the primary function of iron capture and storage in haemolytic diseases, other functions include antigen trapping and presentation to lymphocytes. Sequestration of products of cellular degradation and potentially toxic tissue materials are among the other functions that have been described (Agius and Roberts, 2003).

Dense reticular-like cells recognised as barrier cells in the head kidney of *C. batrachus* appear as pericytes or lining the blood sinusoids resembling those described in the spleen and bone marrow of mammals (Weiss and Geduldig, 1991). The processes of the barrier cells extending among the extracellular matrix may increase the filtration capacity and splenic clearance of blood. Furthermore, barrier cells in the *C. batrachus* haemopoietic tissue are observed closely associated with clusters of haemopoietic and lymphopoietic cells. It thus forms part of the haemopoietic microenvironment, with the function of isolating putative stem cells, concentrating haemopoietic factors and regulating the migration of blood cells in circulation (Weiss and Geduldig, 1991). As such, the presence of the barrier cells in the fish head kidney reinforces the proposed homologies between this organ and the mammalian bone marrow.

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