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Hemopoiesis in the pronephros of tench, *Tinca tinca*, Linnaeus 1758 (Teleostei, Cyprinidae): cytochemical identification and cell morphology

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Abstract: Hemopoiesis in the tench (*Tinca tinca*: Teleostei, Cyprinidae), a cyprinid fish that can be found within the entire territory of Eurasia, from France in the west to the Yenisei River in the east, has not yet been described. The aim of this research was analysis of hemopoiesis lineages in the pronephros of *Tinca tinca* (sampled from the Jablanicko Reservoir). Identification and cells measurements were done by using a light microscope (Olympus BX41) and an Olympus DP12 camera. Hemopoietic cells of the tench are divided into five different series that include erythropoietic, granulopoietic, lymphopoietic, thrombopoietic, and monopoietic cell series. Erythropoietic series consist of a minimum of three stages: basophilic erythroblasts, acidophilic erythroblasts, and mature erythrocytes. Erythroblasts are nucleated and the most abundant hemopoietic cells in the pronephros of tench. Granulopoietic series consist of granuloblasts, namely two different types: neutrophilic granuloblasts (most frequent) and pseudoeosinophilic or heterophilic granuloblasts (POX-positive). Lymphopoietic series consist of lymphoblasts and also small, medium, and large lymphocytes. Thrombopoietic series consist of prothrombocytes and mature thrombocytes, the smallest hemopoietic cells of the tench. Monopoietic series consist of only one maturation stage called monocyte precursor cells, vacuolated and the largest hemopoietic cell in the pronephros of tench. It can be concluded that the pronephros represents a principal place of hemopoiesis in tench.

Key words: Identification, hematopoietic stages, peroxidase activity, head kidney, fish hematology

1. Introduction

Lymphomyeloid (hemopoietic) tissue, specifically distributed in the organism, forms a lymphomyeloid or hemopoietic complex that takes up 0.5%–1.7% of body mass in fish (1). Hemopoietic tissue in hagfish is located in the intestinal submucosa and pronephros, where the intestinal submucosa is mainly granulopoietic while the pronephros is mainly lymphopoietic or granulopoietic (2). Hemopoietic specification of the genus *Chimaera* shows partial hemopoiesis in the cranial and clavicular region. Granulopoietic tissue of sturgeon is located in the posterior part of the meninx; a depression or canal in the posterior part of the cranium shows where extinct Devonian fish were producing blood cells (2).

The kidney is an adult hemopoietic organ, equivalent to bone marrow in mammals; it contains progenitor cells that show the same markers as embryonic stem cells in caudal hemopoietic tissue (3). In kidney marrow, hemopoietic stem cells occupy the space along the epithelia of urinary tubules. Definitive hemopoietic cells derive from the hemogenic endothelium and are capable of directing their differentiation in various hemopoietic lineages (4). On the other hand, other scientific sources claim that mesenchymal cells located ventrally to the dorsal aorta are capable of modulating the generation of stem cells in the aorta-gonad-mesonephros (AGM) region, using many transcription factors such as runx1 (5).

The pronephros is the main hemopoietic organ in fish (6). The intensity of hemopoiesis in the pronephros varies among different fish species. In some species it is entirely erythropoietic but in the other taxa it can be a place of complete hemopoiesis (7). On the basis of many histological studies of the Antarctic fish pronephros (8), the general prevailing conclusion is that the shape of erythrocytes and the number of granulocytes and lymphocytes are in connection with very low water temperature; this phenomenon is considered to be a very effective adaptation of the pronephros as the main immune organ.

Erythropoiesis is regulated with a few very complex factors (scl, GATA family of transcription factors, erythropoietin, erythropoietin receptors, JAK kinase, STAT signal molecules), which modulate the process of erythrocyte maturation (9). Expression of myeloperoxidase is the first sign of emerging granulocytes the second day after the fertilization of zebrafish (10).

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Even in the early stages of embryogenesis, neutrophilic granulocytes participate in sterile acute inflammation (6). In many marine fish, the pronephros contains erythroid, granuloid, lymphoid, and thromboid lineages (11). The pronephros of *Sparus aurata* contains all the maturation stages of erythropoiesis, with all the structural changes clearly visible during the maturation process, such as chromatin condensation and hemoglobin accumulation. Granulopoiesis in the pronephros of *Dicentrarchus labrax* takes place through specific stages: promyelocytes, myelocytes, metamyelocytes, and three types of mature cells – eosinophilic, basophilic, and heterophilic myelocytes/granulocytes (7).

Production of blood and blood cells during embryogenesis occurs in two major waves (12). In zebrafish (*Danio rerio*), the primitive hemopoietic wave consists of forming primitive erythrocytes (GATA1+) in intermediate cell masses, that is, the region of the posterior lateral mesoderm (13) and the primitive myeloid cells (PU.1+) from anterior lateral mesoderm cells (14).

The first primitive wave of embryonic hemopoiesis is equivalent to the blood forming in the yolk sac of higher vertebrates, while the other hemopoietic wave represented by appearance of erythromyeloid progenitors in posterior blood islands located in the caudal embryonic region (15) initiates definitive embryonic hemopoiesis in the AGM region, along the ventral wall of the dorsal aorta (16). AGM cells migrate to the caudal hemopoietic tissue and their further migration progress depends on the kidney and thymus development (17). The abovementioned secondary hemopoietic tissues provide a specific hemopoietic niche or microenvironment for all blood progenitors and enable their proliferation and differentiation.

The aim of this work is to identify the pronephros function in hemopoiesis and provide morphological identification with basic characteristics of hemopoietic cells in the tench.

2. Materials and methods

2.1. Site

The research area is the Jablanicko Reservoir on the Neretva River (Bosnia and Herzegovina). The reservoir spreads from the Konjic municipality to the municipality of Jablanica (43°41′0″N, 17°51′0″E) with an area of 13 km^2 at 270 m a.s.l.

2.2. Sampling and experimental design

Fishnets (Attwod Fold-N-Stow) were used in the sampling. The sample consisted of 35 specimens, 21 female and 14 males, with an average weight of 86.69 g (WBW digital scale). They were transported to the laboratory in containers supplied with constant aeration of water. The adaptation of fish in the laboratory environment lasted 20 days (Laboratory of Physiology, Faculty of Science, University of Sarajevo, Sarajevo, Bosnia and Herzegovina) and included daily monitoring of water exchange, oxygen concentration (Winkler method), and ammonia concentration (Nessler method). Aerators (CHAMPIONCX-0098) were used for water aeration. Fish were fed with Eco FeedEx C 48/10 (Eco Feed Ltd., Serbia).

2.3. Kidney biopsy and hematological methods

Fish hemopoiesis has been studied sporadically and there are no unified methods developed for the biopsy of fish kidney. Prior to the kidney biopsy, fish were euthanized with 0.2% tricaine (Penta Chemicals), placed in ice-cold water for 4 to 5 min, and then dissected, with the digestive tract removed and the kidney carefully separated from the body wall. Approximately 0.5 cm^3 of tissue was taken from the head kidney or pronephros using biopsy tweezers. The sample was used for preparation of touch slides, created by slight zigzag rolling movements of tissue over the slide using a glass rod or pin. Smearing the sample over the slide by imprint method enables the determining of tissue cellularity (18). Following their drying, tissue smears were subjected to the Pappenhein staining method (Penta Chemicals), having been previously treated by the Leders method (Penta Chemicals) to prove the presence of peroxidase activity in granuloblast cells.

2.4. Microscopic analysis

Identification and all measurements of hemopoietic cells were performed using a light microscope (Olympus BX41) connected to an Olympus DP12 camera. Morphological analysis and identification included 300 cells from each hematopoietic lineage.

2.5. Statistics

Data are presented as means ± 1 SD accompanied by minimum, maximum, and coefficient of variation (CV, %). Analyses were performed using SAS 9.0 (SAS Institute Inc., Cary, NC, USA).

3. Results

Based on the light microscopic analysis of touch slides of the tench pronephros, hemopoietic cells identified by morphological characteristics were classified into the abovementioned groups: erythroblasts, granuloblasts, lymphoblasts, neutrophilic granuloblasts, pseudoeosinophilic granuloblasts, prothrombocytes, and monocyte cell precursors, as evidenced in Figures 1a–1f. Dimensions of hemopoietic cells are presented in the Table with summarized mean ± SD, minimum, maximum, and CV values for all measured hemopoietic cells.

In the pronephros of tench, erythroblasts are the dominant hemopoietic cells. They can be observed in different stages of maturation, and the differences among them are based on various nuclei size, ratio between nucleus and cytoplasm, or chromatin condensation. There are two main stages in tench erythropoiesis: basophilic

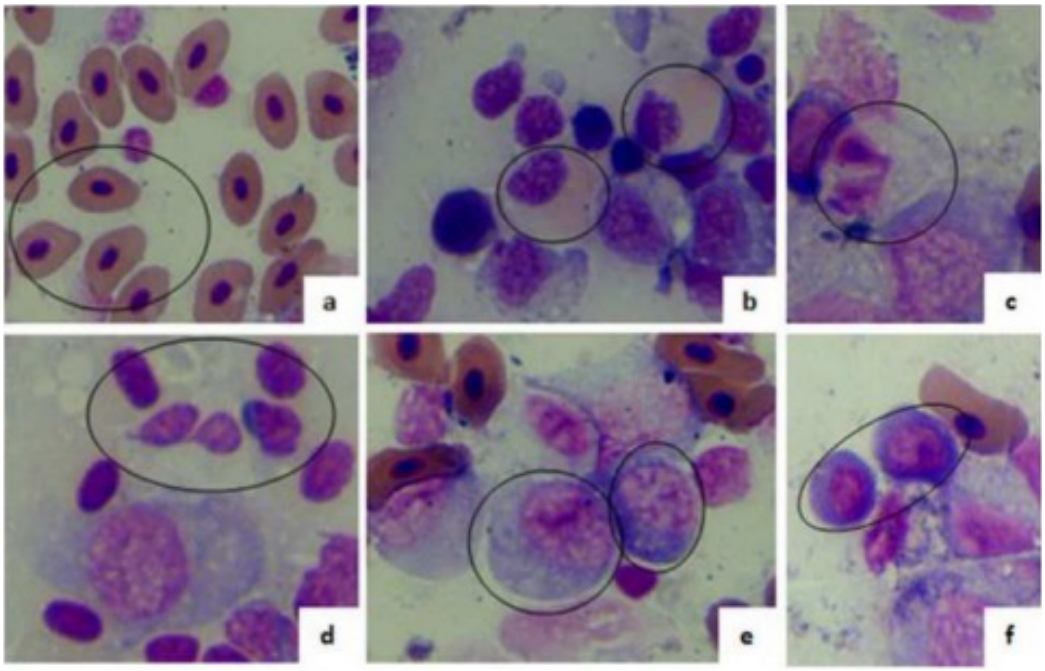

Figure. Hemopoietic cells of *Tinca tinca*: a) erythroblasts; b) POX-positive neutrophilic granuloblasts; c) pseudoeosinophilic granuloblasts; d) prothrombocytes and large monocyte precursor; e) granuloblasts; f) lymphoblasts.

erythroblasts (with darker cytoplasm, larger nucleus, and the presence of inclusions) and acidophilic erythroblasts (acidophilic and lighter cytoplasm and smaller nucleus). Interesting is the appearance of erythroblasts with peroxidase activity. The breadth of erythroblast nuclei was very variable (CV = 21.06%).

Granuloblasts are large cells (13.64 µm length; 11.99 µm breadth), with large and eccentric nuclei that occupy most of the cytoplasm. The cytoplasm of these cells is basophilic. The cells are round or irregularly shaped. All observed granuloblasts were peroxidase-negative as enzyme synthesis had just begun.

Lymphoblasts, in addition to the prothrombocytes, are the smallest cells in the pronephros of tench (8.52 µm length; 7.68 µm breadth). Lymphoblasts are round cells, with more than 80% of the cytoplasm being occupied by the nucleus. The cytoplasm is agranulated with intensely blue coloration. All lymphoblasts are POX-negative and do not show an affinity to peroxidase. Cell length (16.79%) and breadth (17.37%) in lymphoblasts were very variable.

Neutrophilic granuloblasts are the most common form of leukocyte. As they mature, the ratio between the cytoplasm and nucleus changes in favor of the cytoplasm, where the nucleus becomes eccentric with gradual nuclear lobulation and segmentation. The cytoplasm is brightly colored with neutrophilic granules.

Pseudoeosinophilic granuloblasts, in addition to prothrombocytes, are the most numerous leukocytes in the pronephros of tench. During the maturation process, the nucleus size constantly decreases, its shape resembling a flattened ball, and it increasingly clings to one side of the cell membrane. The cytoplasm contains a large number of secondary granules, often causing a vacuolated appearance of the cells. Pseudoeosinophilic granuloblasts contain myeloperoxidase in the cytoplasm, which is not yet stored in lysosomal granules and therefore exhibits less activity.

Prothrombocytes are the smallest cells of hemopoiesis in tench (5.57 µm length; 5.01 µm breadth). Morphologically, prothrombocytes are most similar to the cells of the lymphocyte lineage. The nucleus of the prothrombocytes occupies more than 95% of the cytoplasm and has deep violet coloration. In almost all prothrombocytes, only the edge of the cytoplasm is visible. Prothrombocytes never contain peroxidase.

Precursor cells of monocytes are the largest cells in the hemopoietic tissue or pronephros in tench; they were 16.79 µm in length and 14.46 µm in breadth. These cells have a very large nucleus, which takes up more than 50% of the cytoplasm and is always eccentric. The cytoplasm is usually filled with small vacuoles. Monocyte precursor cells never contain peroxidase.

4. Discussion

Previous studies of fish hematology are very obscure and sporadic, especially in relation to hemopoiesis. The reason could be the fact that in different fish species, different

Table. Basic measurements of hemopoietic cells in tench.

Type of cell	Cell/nucleus	Parameter	Mean ± SD	Minimum	Maximum	CV (%)
Erythroblasts (100)	Cell (μm)	Length	13.22 ± 1.26	11.28	15.48	9.50
		Breadth	7.69 ± 0.58	5.79	8.47	7.51
	Nucleus (μm)	Length	4.00 ± 0.50	3.13	4.77	12.49
		Breadth	2.61 ± 0.55	1.65	3.77	21.06
Neutrophilic granuloblasts (80)	Cell (μm)	Length	12.73 ± 1.05	11.68	15.97	8.29
		Breadth	11.44 ± 1.09	9.11	13.72	9.55
	Nucleus (μm)	Length	9.30 ± 1.03	7.87	11.79	11.02
		Breadth	7.61 ± 0.85	6.10	8.84	11.01
Pseudoeosinophilic granuloblasts (80)	Cell (μm)	Length	13.54 ± 1.04	11.63	15.31	7.58
		Breadth	12.57 ± 0.91	11.27	14.51	7.25
	Nucleus (μm)	Length	9.07 ± 1.16	7.56	11.27	12.84
		Breadth	6.73 ± 1.32	4.52	8.75	19.55
Granuloblasts (60)	Cell (μm)	Length	13.64 ± 1.71	11.27	17.01	12.53
		Breadth	11.99 ± 1.56	9.22	15.28	13.00
	Nucleus (μm)	Length	9.16 ± 1.10	7.26	11.04	11.98
		Breadth	7.72 ± 1.10	6.14	10.02	14.28
Lymphoblasts (60)	Cell (μm)	Length	8.52 ± 1.43	6.46	10.49	16.79
		Breadth	7.68 ± 1.33	5.80	10.29	17.37
	Nucleus (μm)	Length	6.29 ± 0.97	4.87	8.73	15.41
		Breadth	5.47 ± 0.55	4.59	6.60	10.05
Prothrombocytes (70)	Cell (μm)	Length	5.57 ± 0.44	4.68	6.30	7.94
		Breadth	5.01 ± 0.45	4.31	5.64	9.05
	Nucleus (μm)	Length	5.22 ± 0.55	3.37	5.10	13.00
		Breadth	3.95 ± 0.53	3.01	4.56	13.36
Monocyte precursors (25)	Cell (μm)	Length	16.79 ± 1.34	15.18	18.45	7.97
		Breadth	14.46 ± 0.75	14.54	15.57	5.17
	Nucleus (μm)	Length	11.33 ± 0.89	10.57	12.59	7.82
		Breadth	9.61 ± 0.55	9.15	10.34	5.77

Number of observed cells is given in parentheses; CV: coefficient of variation.

hemopoietic sites produce hemopoietic cells, such as the Leydig organ, epigonal organ, intestinal submucosa, throat wall, cranial fillister, pronephros, spleen, thymus, and liver. Hemopoietic tissue makes a hemopoietic complex that occupies 0.5%–1.7% of the body mass in fish (1). On the other hand, the site of hemopoiesis and cell maturation is very mutable, even in different species of the same taxonomic family, which is the reason why there is still no uniform classification of hemopoietic cells in fish.

The pronephros is well supplied with blood, due to the renal portal circulation, and is considered a major hemopoietic organ in teleostean fish (6). The pronephros contains a very small number of excretory tubules and a large number of hemopoietic cells (11). Our study shows

that the pronephros is the major hemopoietic site in tench. In the pronephros of tench, several hemopoietic cells can be detected with obvious differences, based on the available literature (6,7,19).

Erythropoiesis is characterized by one starting cell, which during the maturation process passes through certain morphologic changes, including the change of the ratio between the cytoplasm and nucleus, cytoplasmic staining, and chromatin condensation (19,20). If these results are compared with mammalian erythropoiesis, in which cells undergo separate stages of maturation, it can be concluded that this phenomenon is unique only in a few fish species. Erythropoiesis in tilapia (*Ochreomis niloticus*) includes basophilic, polychromatophilic, acidophilic erythroblasts and immature and mature erythrocytes (21). During erythropoiesis in tench, all maturation stages show great similarity in relation to the size of the nucleus, cytoplasmic staining, and cell shape. In all erythroblasts of tench, a very gentle transition can be observed in cytoplasmic staining, pointing to the existence of transitional cell forms such as basophilic, polychromatophilic, and acidophilic erythroblasts (22,23). The initial erythropoietic cells have a large nucleus and darker cytoplasm; however, these cells are quite similar to mature cells, which demonstrate a great ability of fast mobilization from the pronephros to peripheral circulation. This phenomenon occurs due to the increased activity of fish or due to a lack of oxygen in water; this phenomenon would occur much more slowly if the morphological differentiation followed separate stages of erythropoiesis. This feature is associated with good adaptive mechanisms. Erythroblasts make up 1/3 of all hemopoietic cells in tench.

The granulopoietic lineage in tilapia (21) consists of the starting granuloblast, progranulocyte, metagranulocyte, and mature granulocyte. In tench, various stages of granulopoiesis were detected. We have established different stages of granuloid cell maturation, which is especially reflected in the staining of the cytoplasm and the presence of specific and nonspecific granules. Granuloblast cells appear in small numbers due to their high potential of division and differentiation (23). Granuloblasts, as very large cells, by the maturation process give rise to neutrophilic and pseudoeosinophilic granuloblast or heterophils.

Pseudoeosinophilic granuloblasts are a specific characteristic of hemopoiesis in tench. The reason for a lack of other granuloid cells remains unknown. Pseudoeosinophilic granuloblasts are the most abundant granuloid cells containing neutrophilic, eosinophilic, and basophilic granules, which may be the reason for the lack of basophilic and eosinophilic granuloblasts (24). Pseudoeosinophilic granuloblasts are the only peroxidase-positive cells in the pronephros of tench, which helps their differentiation from other granulopoietic cells. A large number of pseudoeosinophilic granuloblasts indicate a quick adaptive reaction to stress, because pseudoeosinophilic granulocytes are also very abundant in peripheral circulation (25). The peroxidase-positive granulocytes appear in the embryogenesis process, which indicates their role in the adaptive immune reaction (10).

Lymphocytopoiesis includes several stages of cell maturation. Lymphoblasts are generally described as cells with voluminous nucleus, small cytoplasm, and several large mitochondria. In lymphoblast analysis, two distinct forms have been observed, i.e. large and small lymphoblasts (26). The existence of these cells in the pronephros of tench is unquestionable and implies a rapid and adaptive immune response in tench, which makes it evident that tench is a very resistant fish.

Macrophages (monocytes) in tilapia are the cells that have been observed mainly in melanomacrophage centers of pronephros, whereby it is assumed that the pronephros is their place of origin (21). Cells precursors of monocytes in tench are the largest cells with eccentric nuclei and vacuolated cytoplasm (27). Apart from the kidney, monocytes of tench can be observed in the peripheral blood (25). Monocyte precursor cells are few in number, which certainly correlates with their function, and also with the assumption that the place of monocyte emergence may still not be described with certainty.

The size of hemopoietic cells in tench is quite interesting and these are the first data relating to their size. Prothrombocytes, as the only stage described in thrombopoietic series, exist in large numbers, which was previously confirmed (21). These cells are characterized by a pronounced hemostatic and partially unexplored phagocytic activity. Prothrombocytes contain nuclei and are classified as leukocytes (23,27). Prothrombocytes also appear in two shapes: round and elliptic. The comparison of prothrombocytes of tench and rats shows a main difference in the process of maturation, i.e. in rats thrombocytes emerge from the largest hemopoietic cell (megakaryoblasts) and become the smallest blood element or platelets without nuclei, while in tench, thrombocytopoiesis occurs in one or two maturation stages with very similar morphological features (28).

The absence of morphologically different stages of hemopoietic cells in tench is probably an advantage in conditions of rapid adaptive response to any stress reaction. The speed of adaptive response is reflected in the function of blood cells, namely erythroblasts and heterophils. A very rapid maturation of erythroblasts can ensure the supply of oxygen, which is associated with respiratory motion, mobility, and energy metabolism. The role of pseudoeosinophilic granulocytes, neutrophils,

basophils, and eosinophils is a powerful factor in the immune defense.

Based on the light microscopic analysis of touch slides of the tench pronephros and numerous scientific analyses of hemopoietic cells and peripheral blood of mainly salmonid and cyprinid fish, it can be concluded that the pronephros represents a principal place of hemopoiesis in tench. The cells identified by morphological characteristics were classified into the abovementioned groups: erythroblasts, granuloblasts, lymphoblasts, neutrophilic granuloblasts, pseudoeosinophilic granuloblasts, prothrombocytes, and monocyte cell precursors. The smallest hemopoietic cells in tench were prothrombocytes while the largest were monocyte precursor cells.

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