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REVIEWS

Peculiarities of Organization and Functioning of the Fish Red Blood System

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Abstract—The review presents information on localization of sites of erythropoiesis in the fish organism, morphological peculiarities and proliferative activity of erythroid elements at different stages of differentiation, and life span of circulating erythrocytes. Data about effects of various factors on production of erythrocytes by hemopoietic tissue, such as erythropoetin and several other biologically active substances, are presented. Peculiarities are considered of organization of fish spleen as an organ storing and destroying old erythroid forms. Significance of production and destruction processes in the red blood system in correction of erythrocytic homeostasis in the fish organism is discussed.

INTRODUCTION

Circulating blood and hemopoietic tissue of marine and fresh-water fishes has a subject of studies for as long as 70 years. By the present time, extensive information has been accumulated on these problems. However, the studies performed for the last 15 years have extended significantly our concepts. There have been obtained data about peculiarities of humoral regulation of erythropoietic processes, processes of aging and destruction of red blood cells, blood depots, life span of circulating erythrocytes, etc. All this allows approaching consideration of the fish red blood as of a physiological system. Such approach is used as the basis for the present review. It considers, alongside with evaluation of the state of the functional chain, the circulating blood, also the main chains of regulation of erythrocytic homeostasis: erythropoiesis, destruction of old erythroid forms, blood depots, which have not been included into the previous reviews.

CIRCULATING BLOOD

Quantitative values of hemoglobin concentration and the erythrocyte count in the fish blood have pronounced species specificity. There are species both with extremely low values of these parameters (erythrocytes—$0.5–1.5 \times (10^{12})$ l^{-1}; hemoglobin—50–70 g/l) [8, 9] and with extremely high values (erythrocytes—$3.0–4.2 \times (10^{12})$ l^{-1}; hemoglobin—125–130 g/l) [10, 11]. These differences are mainly due to the level of the fish natural motility. Pelagic, actively migrating species practically always have a high blood oxygen capacity. On the contrary, circulating blood of benthic, low-active species has a low level of the pigment and the low number red blood cells. This regularity has been reported in papers of many authors [12–14]. Some Antarctic fish species that combine a low motor activity and habitation in a supercooled environment (–1.8°C) have no hemoglobin and erythrocytes in blood at all [15].

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Quantitative parameters of fish red blood are affected essentially by swimming load. Thus, in the flying fish *Exocoetus volitans* the number of red blood cells at the moment of flight reaches 3.0–$4.2 \times (10^{12})$ l^{-1} [8]. Concurrently, elevation of hematocrit values and swelling of circulating erythrocytes is observed [16]. Similar effects are produced by the stress due to capture and transportation [6, 17]. This response is characteristic of representatives of pelagic ichthyofauna. In benthic fish, swimming often produces opposite changes [18].

Spawning is commonly accepted to be a peculiar "pivot" of most metabolic processes in the fish organism in the course of annual cycle. The state of physiological systems at this period experiences the most essential changes. The red blood is not an exception. It has been found that oxygen capacity of blood at the pre-spawning period and in the beginning of the spawn decreases substantially. Hemoglobin concentration and the number of erythrocytes in blood fall [19, 20]. On the contrary, by the end of spawning and at the post-spawning period (for 2–3 months) a marked increase of the blood oxygen capacity is observed, which is indicated by an elevation of hemoglobin concentration and of the number of circulating erythrocytes [19–21]. Such dynamics of the red blood state is not characteristic of all fish species. Some individuals, even within the same biological species, may have both an increase and a decrease of the blood oxygen capacity at the pre-spawning period. In some cases, the changes may be poorly expressed [22]. During preparation of the fish organism for spawning, an essential redistribution of plastic resources is known to occur. This is a basis for suggestion that changes in response of peripheral blood depend on the preceding nutrition regime of individuals [23]. If it is insufficient, plastic resources of other physiological systems, particularly of circulating blood, are used, and *vice versa*.

Alongside with biotic factors, the fish blood oxygen capacity is affected essentially by environmental conditions. The most important of them is temperature. The character of its effects is determined by limits of temperature tolerance of the species. In the range of stability there is a direct relationship between the environmental temperature and the blood oxygen capacity. An increase of blood hemoglobin concentration and the number of erythrocytes coincides with intensification of organism metabolic processes [24–26]. An opposite pattern is observed in the zone of low temperatures [27]. Positive correlation between environmental temperature and blood oxygen capacity is disturbed outside the limits of the species temperature stability. In the zone of high temperatures the blood hemoglobin concentration and the number of erythrocytes decrease, while in the zone of low temperatures it rises [27]. Of special interest are the studies reporting a significant increase of oxygen capacity in winter. This phenomenon has been observed in the carp *Cyprinus carpio* under conditions of pond growing [28] as well as in several marine and fresh-water fish species in the natural environment [27, 29]. The nature of this phenomenon is not completely clear, as increase of hemoglobin concentration was observed at low rates of energy metabolism and coincided with high oxygen content in water.

The oxygen content in environment also affects essentially quantitative parameters of the fish red blood. Extreme variants of hypoxia are accompanied by an increase of the number of erythrocytes, hemoglobin concentration, and hematocrit in peripheral blood [12, 24], the rise of hematocrit values clearly exceeding that of the rest of the parameters. This is to the great extent due to swelling of circulating erythrocytes [30]. If the decrease of oxygen concentration in environment does not reach extreme values, the above reaction can be either poorly expressed or absent altogether [31].

It follows from the presented data that oxygen capacity of fish blood is a very labile parameter that reflects sufficiently well changes of the functional state of individuals and their adaptation to environmental conditions. This implies the existence in their organism of very effective functional mechanisms of correction of the red blood quantitative parameters. These mechanisms can be based on three processes:

(1) a change of ratio of production (erythropoiesis) and destruction processes in the red blood system; (2) activation of a large mass of erythrocytes replaced from circulation or its reserving (blood depot); (3) hydration or dehydration of blood plasma (relative changes).

LOCALIZATION OF SITES OF ERYTHROPOIESIS

Functional analog of red bone marrow in teleost fish is considered to be the anterior part of kidney (pronephros). This has been shown convincingly for most of species of this taxonomic group of organisms [32–34]. Analysis of pronephros histological structure has revealed the presence in it of reticular cells and reticuline fibers. Melanomacrophagal elements also are well-formed here [33]. The sizes of erythropoiesis focus in the head kidney are variable and submitted to season fluctuations [32]. The most active proliferation of erythroid cells in the teleost fish head kidney occurs at the larval period of ontogenesis. In adults, the mitotic index among immature erythroid forms usually does not exceed 1% [32].

Alongside with pronephros, the middle part of kidney (mesonephros) also participates actively in production of erythrocytes [35]. The mass of the erythroid cell population in it in some species exceeds that in other sites of hemopoiesis. It is to be noted that blood stem cells are anladed in mesonephros as early as at the 4th day after hatching. However, in pronephros this occurs somewhat earlier (at the 2nd day) [36].

Participation of spleen in erythropoiesis is disputed in many works. Some authors consider it as an organ, in which only processes of lympho-, thrombo-, and granulopoiesis occur [34, 37]. Thus, splenectomy produces no obvious changes in peripheral blood in the rainbow trout *Oncorhynchus mykiss* [38]. In some species such intervention was accompanied by development of hypochromic type anemia [39]. However, this rather reflects the fact that spleen is a source of hemosiderin needed for normal synthesis of hemoglobin, but not an organ of erythropoiesis [5, 40]. At the same time, erythroid elements at various differentiation stages were revealed in spleen of several marine and freshwater fish species [41, 42]. Analysis of histostructure showed their spleen to have similar features of organization with spleen of the higher vertebrates [37]. The erythroid anlage inside is located predominantly in the red pulp [43]. Erythropoiesis in this organ was found to be less intensive than in the kidney. On this basis, it was suggested that spleen played more likely a role of secondary site of erythropoiesis. Erythroid precursors migrate into the spleen at the moment when the size of the erythropoiesis focus reaches critical level [44]. The most active proliferation of erythroid elements was revealed in spleen of teleost fish only at early stages of ontogenesis [36, 45]. These processes were suppressed with age.

Unlike teleost fish, spleen in cartilaginous fish is the major organ of erythropoiesis. It is here where all anlages of hemopoiesis including erythropoiesis are concentrated. Involvement of kidney in the hemopoietic processes in this taxonomic group is rather weak [35].

Alongside with kidney and spleen, erythropoietic tissue was also found in several other organs. Of special importance is endothelium of heart [39] and blood vessels [41]. Unilateral nephrectomy has been shown to activate significantly erythropoietic processes in the heart [39]. Foci of erythropoiesis in blood vessels were observed mainly at early stages of embryogenesis. Erythropoietic tissue at this period is also present in liver and intestine of embryos [46]. Rather unexpected are data about the presence of erythroid cell populations in thymus of trout [47]. The authors discuss possible role of microenvironment and hormonal factors providing erythropoiesis in the organ.

PROLIFERATIVE ACTIVITY AND DIFFERENTIATION OF ERYTHROID ELEMENTS

Erythroid line of marine fish cells is represented by the following differentiation stages: stem forms → erythroblasts → proerythroblasts → basophilic normoblasts → polychromatophilic normoblasts → acidophilic normoblasts → reticulocytes → mature erythrocytes [33, 48]. Previously, the term "hemocytoblasts" was used instead of "stem cells." At present it is practically not used. The stage of erythroblast also is not identified in all studies, which is due to certain difficulties of its morphological identification. Therefore, the erythroid line is usually started from proerythroblast. It is also considered as a morphological analogue of blood stem cells. Due to uncertainty of morphological signs the stage of reticulocyte also is identified sufficiently seldom.

The process of differentiation of erythroid forms includes their following morphological changes: a decrease of cell sizes, acquisition of ellipsoid shape, condensation of nuclear chromatin and reduction of nuclear sizes, a decrease of the number of free ribosomes in the cytoplasm and their organization into polyribosomes, and change of the cytoplasm color from blue to pink tints, which is due to accumulation of hemoglobin inside it [26, 48–50]. Ultrastructural peculiarity of mature fish erythrocytes also is preservation in their cytoplasm of large single mitochondria, Golgi complex, and vacuolar vesicles [50–52]. These changes have been shown to occur under control of transcription factor SCL/Tal-1 [53]. Duration of the process of differentiation and maturation of erythrocytes in marine fish is different and depends on the organism functional state. Thus, in adult individuals of Baikal salmon it is as long as 41 days [54], while under conditions of acute hypoxia it can be shortened to 14 days.

Proliferative pool of fish erythroid anlage is represented by stem cells, proerythroblasts, and basophilic normoblasts. They intensively incorporate ^{3}H-thymidine and are located in pronephros and spleen [55]. It has been shown that renal stroma actively absorbs proliferative forms [32] and is able to control direction of differentiation of stem cells [56]. Peripheral blood usually contains cells not capable for active proliferation, such as late basophilic normoblasts as well as polychromatophilic and acidophilic normoblasts [41]. However, under conditions of intensive erythropoiesis, less differentiated erythroid forms can appear in peripheral blood: pronormoblasts and early basophilic normoblasts able to divide [51, 57]. Cases of amitotic division of mature erythrocytes, which is induced by external hypoxia, also are described [58, 59].

REGULATION OF ERYTHROPOIESIS

Erythropoiesis in higher vertebrates is regulated by specific biologically active substances, erythropoietins. They are acidic glycoproteins of molecular weight of 46–60 kDa, produced by kidney. They control rates of proliferation and differentiation of erythroid cells. The most sensitive to them are late erythroid committed precursors.

First evidences for the existence of erythropoietic factors in the fish blood were obtained in experiments on kissing gourami [60]. Administration to starved individuals of the blood plasma from fish exposed to hypoxia induced erythropoiesis in the hemopoietic tissue. Similar effect was produced by kidney and spleen homogenates obtained from animals submitted to anemia [61]. Hemopoietic tissue of fish was sensitive to erythropoietins of humans and other vertebrates. Processes of proliferation and differentiation of erythroid elements were enhanced after administration of standard erythropoietin preparations. The total production of erythrocytes by hemopoietic tissue increased [60]. Many authors have reported the starved individuals to be a convenient experimental model for comparative testing of erythropoietic activity of blood plasma of other fish [60]. The technique developed on this basis has allowed assessing a relative level of erythropoietins in blood of benthic and pelagic species [62]. In low-active fish maintained under similar conditions it turned out to be higher by 30–90%.

By analogy with higher vertebrates, it was assumed that erythropoietic factors in the fish organism were produced in kidney. One of the first comparative studies carried out on the bullhead *Ameiurus nebulosus* has confirmed the suggestion. Kidney homogenates from animals submitted to anemia produced more pronounced effect on the fish hemopoietic tissue in comparison with spleen [61]. However, the ultimate proof in favor of the presence of erythropoietin in the fish organism was obtained in experiments on rainbow trout. This substance was identified immunohistochemically in kidney, spleen, liver, and blood plasma of this species [1], with the highest concentration of erythropoietin being found in kidneys.

Production of erythropoietin and intensity of erythropoiesis is also affected significantly by the fish state. The most dramatic changes are observed during spawning period and are due to the level of sex hormones in blood. Thus, a rise of testosterone level in plasma at the period of maturation of gonads has been shown to be accompanied by an increase of erythropoietin production in kidneys and to results in activation of erythropoiesis in hemopoietic tissue [63]. Similar effect has also been reported for melatonin [64].

ERYTHROPOIESIS AND ENVIRONMENTAL FACTORS

Positive correlation has been found between intensity of erythropoiesis in fish and environmental temperature [44]. It is accompanied by a shortening of the mitotic cycle and enhancement of proliferation in the erythroid cell line. Index of ^{3}H-thymidine incorporation increases. However, these changes are usually observed in the range of the species tolerance temperatures. With the temperatures outside this range, erythropoietic processes are suppressed [26] and their sensitivity to controlling signals (blood pO_2) decreases [24]. Some authors report that the environmental temperature rather produces an indirect effect on the hemopoietic tissue via alimentary activity of the species [42].

Various variants of experimental hypoxia and anemia (phenylhydrazine-HCl) also activate proliferation of immature erythroid elements and production of erythrocytes by hemopoietic tissue [26, 44, 54]. Cells with morphological signs of erythroblasts appear in the circulating blood. Intensity of ^{3}H-thymidine incorporation increases both in the peripheral circulation and in hemopoietic tissues (kidneys, spleen) [55]. Hypercapnia also increases the rates of erythroid cells division, but has no effect on the process of their maturation [54].

Processes of erythrocyte formation in the fish hemopoietic tissue are submitted to pronounced seasonal fluctuations. No general regularities have been revealed. The obtained data are rather species-specific and usually do not reflect natural dynamics of water temperature [47]. The most significant changes occur at the spawning period. Erythropoietic processes this period in most fish are almost completely suppressed. However, at the post-spawning period a sharp activation of production processes occurs and lasts for several months [19, 20]. Mechanisms underlying this phenomenon are not ultimately elucidated.

DESTRUCTION OF OLD ERYTHROID FORMS

In fish, like in the higher vertebrates, destruction of old erythroid forms takes place in spleen. It is the main site of storage of old erythrocytes [4]. The hemosiderin level is the highest here (8.6%) as compared with liver and kidney [5, 40]. This protein performs iron transport to hemopoietic tissue after degradation of hemoglobin molecule. Splenic melano-macrophagal elements participate actively in this destruction [42, 65]. The most active are macrophages of the red pulp.

With aging of fish erythrocytes, dramatic changes in their metabolism occur. They involve aerobic and anaerobic cell metabolism as well as processes of protein biosynthesis. Oxygen consumption by erythrocytes falls more than twice. This occurs on the background of suppression of activity of several key enzymes: cytochrome oxidase, citrate synthase (by 35–100%), and lactate dehydrogenase (by 70%) [2]. This is accompanied by dehydration of the cell cytoplasm, which is indicated by a decrease of erythrocyte volume [2]. Protein production decreases by 50%, while the total RNA level falls 10 times [24].

Loss of elastic properties of the membrane of circulating erythrocytes is the main cause of their retention and subsequent destruction in spleen. This can be a consequence both of natural aging processes and of changes of cell resistance for various reasons. Study of the fish erythrocyte resistance to osmotic shock has shown this parameter to depend on quite a few factors. One of them is the qualitative content of dietary lipids [66]. An elevated content of oxidized lipids in the mixed feed produces a negative effect and decreases osmotic stability of erythrocytes [67]. Addition of vitamin E to feed has opposite effect [68]. Stress induced by catching and transportation also affects cells of the fish red blood. Their resistance to osmotic shock falls [69]. A decrease of osmotic resistance of erythrocytes was also observed after increase of NH_4^+ concentration in water [70].

LIFE SPAN OF CIRCULATING ERYTHROCYTES

Intensity of the production and destruction processes in the fish red blood system can be evaluated from the erythrocytes life span. Information of this kind is scarce. The first data were obtained for burbot [71]. The life span of its red blood cells was estimated by maturation rate of polychromatophilic normoblasts in experiments *in vitro*. In satiated fish it amounted to 104 days, while in starved

animals—234 days (2 months of starvation) and 490 days (7 months of starvation). Similar results were obtained with use of ^{51}Cr for fed rainbow trout—105 ± 17 days [72]. Studies performed for one year on the mirror carp with use of ^{3}H-thymidine gave different results [73]. The erythrocyte life span amounted to about 310 days. The first mature cells labeled with ^{3}H-thymidine appeared in the peripheral blood flow as early as at the 6th day and disappeared at the 315th day. Close results, 270 days, were obtained using a fluorescent probe [7]. These data seem to be the closest to the real situation, as intensity of feeding of individuals in natural populations over a year varies and depends on many environmental factors. They agree with results obtained for several marine and freshwater fish, these results indicating the fish hemopoietic tissue to function once a year for several months [19, 20].

RESERVES OF BLOOD DEPOTS

In the higher vertebrates, one of effective mechanisms of correction of blood oxygen capacity is functioning of blood depot reserves. The main mass of the reserved erythrocyte population is located in spleen that participates in providing mechanisms of emergency adaptation by release of stored erythrocytes into the circulating blood.

The first attempts to reveal such mechanism in teleosts turned out to be success. Experiments on the yellowtail *Seriola quinqueradiata* have shown swimming in cruising and maximal regimes to be accompanied by a decrease of the spleen weight and its hemoglobin content [74]. Release of erythrocyte masses from spleen provided 40% of an increase of hemoglobin concentration in blood. Similar effect on the state of this fish organ was produced by hypoxia [60, 75] and temperature stress [6]. Detailed measurements showed that weight of the spleen during the moment of maximal constriction could decrease more than twice, while hemoglobin concentration in spleen could fall by more than 90% [75, 76]. Comparative studies carried out on several Black Sea fish showed reserves of blood depot in representatives of pelagic ichthyofauna to exceed 5 times those in benthic fish [77].

Detailed study of histological structure of spleen in the relaxed and contracted states was performed on the rainbow trout *Oncorhynchus mykiss* [78, 79]. Spleen of this species was shown to have the open circulation system: capillaries ended in reticular network. Terminal arterioles have sphincters that, on one hand, regulate the character of blood flow in the organ, while on other hand, prevent the reverse blood flow at the moment of the organ contraction. Release of the reserved cells occurs exclusively into the venous system. Cells of the reticular network facilitate the decrease of spleen volume. Release of the stored erythrocyte mass at the moment of contraction occurs through a special aperture located in the proximal part of the organ.

Spleen of teleost fish is innervated by cholinergic and adrenergic postganglionic fibers in the celiac nerve. Adrenergic stimulation (experiments *in vitro*) leads to development of resistance to the fluid flow in the perfused spleen [80]. A similar effect is produced by adrenaline administration [76]. Development of the maximal contraction of the organ takes 5 min.

CONCLUSIONS

The complex of the presented data allows several generalizations to be made.

Sites of erythropoiesis in teleosts are concentrated predominantly in pro- and mesonephros, while in the cartilaginous fish—in spleen. The ability to produce erythrocytes is also peculiar to cardiac and vascular endothelium, thymus, and several other organs. Functioning of the hemopoietic tissue varies and is submitted to annual cycle. Proliferative activity of the erythropoietic tissue is controlled by erythropoietins—substances produced by kidney.

Spleen is the site of reserving and destruction of old erythrocyte mass. Tissue of this organ has a high level of iron involved in the structure of hemosiderin. Its state is controlled by cholinergic and adrenergic postganglionic fibers included in the celiac nerve. Spleen is able to release its depot erythrocytes into circulating blood in the case of an increase of organism oxygen needs.

The fish peripheral blood in norm contains erythroid elements that lost their capability for cell division. Turnover of its composition is slower than in the higher vertebrates and is related to alimentary activity of the species. The mean life span of the functional pool cells is close to 300 days.

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