

**EFFECTS OF HYPOXIA ON ERYTHROPOIETIN LEVELS  
IN TROUT KIDNEY AND SPLEEN**

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**Introduction**

Hypoxia is a common event in aquatic environments. Fish respond to hypoxia by attempting to maintain oxygen delivery, down regulating aerobic processes and up-regulating anaerobic metabolism. Fish try to maintain tissue oxygen levels by increasing ventilation, altering the pattern of blood flow and increasing the number of red blood cells (RBC) and haemoglobin (Hb) concentration in the blood (Randall, 1982; Randall and Perry, 1992.).

In most fish, erythropoiesis occurs in the kidney, (Fange, 1986) whereas human erythropoiesis occurs in the bone marrow (Jelkmann, 1992). Erythropoiesis is regulated by the hormone erythropoietin (EPO) in mammals, and probably fish. In mammals, plasma EPO concentrations increase slowly in response to increased levels of hypoxia inducing factor 1 (HIF-1) caused by hypoxic conditions. EPO remains elevated throughout the hypoxic period (Jelkmann, 1992). It is reported that, in rainbow trout kidney, a protein exists that showed competitive binding with antibodies raised against human EPO (Wickramasinghe, 1993). Thus, EPO or an EPO-like molecule has been found at least in a single fish species, the rainbow trout.

There is no information, however, on the effects of hypoxia on EPO production in trout kidney. In this study the effects of hypoxia on renal EPO production in rainbow trout were examined along with changing profiles of EPO levels in the spleen and the plasma.

## Materials and Methods

Rainbow trout, *Oncorhynchus mykiss*, weighing between 350–700 g, were reared at 12°C under natural light conditions for 2 months in an outdoor tank with running dechlorinated Vancouver tap water. During this process, the fish were fed twice a day with commercial trout pellets at a daily amount of 2 % body weight. The fish were transferred to indoor tanks (100 L volume) with running dechlorinated tap water, with 3 fish per tank, under 12 hr light and 12 hr dark, maintained for 2 weeks at same temperature and rearing conditions, and then the hypoxic experiments were performed. Experiments were performed from November to December 1999.

The fish were exposed to two hypoxic conditions, 30% (severe hypoxic condition) and 55% (moderate hypoxic condition) oxygen saturation levels, for 144 and 216 hours respectively. Oxygen levels in the inflowing water were reduced by passing the water down through a column against a flow of nitrogen gas. At the start of the experimental period the tank was covered and the fish were unfed for the exposure period.

Fish were sampled after 4h, 8h, 12h, 24h, 48h, 72h, and 144h exposure to 30% oxygen saturation and after 1, 3, 6, and 9 days exposure to 55% oxygen saturation in the water. A blow to the head was used to stun fish and then blood was collected quickly from the heart using an EDTA-treated syringe. The kidney and spleen were also sampled. Spleen-somatic indices (SSI: [spleen weight/body weight] x 10<sup>4</sup>) were calculated. Hb concentrations were measured by the cyanmethaemoglobin method (Kakuta et al., 1992). Plasma samples were immediately centrifuged at 3,000 g for 10 min at 4°C and then stored at –80°C for later assay.

For EPO assay, the kidney and the spleen were homogenized with 9 volumes of a cold Cortland saline (pH 7.4), containing 0.2 mM phenylmethylsulphonyl fluoride. After centrifugation of the tissue homogenates at 10,000 g for 30 min at 4°C, the EPO levels and total protein concentrations in the supernatants were measured. An aliquot of the plasma samples was concentrated 5 times using a micropore filter to separate substances of *M.W.* below 5,000 and then assayed for EPO using an ELISA kit (Quantikine IVD; R&D systems, USA). Protein was measured using a modified Lowry method (Lowry et al., 1951).

All data in this study were presented as mean±SD. Statistical differences between means of the dependent variables were determined by Duncan's method.

Differences were accepted as significant at the 95 % level of confidence ( $p < 0.05$ ).

## Results

EPO levels in kidney and spleen from rainbow trout kept under normal DO conditions (initial control values) were  $37.1 \pm 8.84$  mIU/100mg protein and  $4.95 \pm 1.34$  mIU/100mg protein, respectively. Thus, the values for renal EPO levels were about 8 times those of the spleen. In rainbow trout exposed to severe hypoxic conditions, renal EPO levels increased significantly at 8, 12, 24, 72 and 144h and at 8, 24 and 144h, compared with those of the initial control and the control groups, respectively. On the other hand, the spleen EPO levels decreased significantly at 8, 12, 24, 72 and 144 hours of hypoxic exposure, compared with control values. In rainbow trout exposed to moderate hypoxia, renal EPO increased significantly at 24, 144 and 216 hours and on 144 and 216 hours of exposure, compared with initial control and control values, respectively. Spleen EPO was higher than that of the initial control value after 144 hours of hypoxic exposure. In comparison with the control, spleen EPO decreased significantly on hour 24 and increased on hours 144 and 216 in fish exposed to moderate hypoxia. No EPO was detected in either concentrated or untreated plasma.

Hb concentrations increased significantly on hours 2, 4, 8, 12, 24 and 48 in fish held under severe hypoxia, compared with the initial and control values. [Hb] increased significantly in fish exposed to moderate hypoxia on hours 24, 72 and 144, compared with initial and control values. SSI decreased significantly on hours 2, 8, 24 and 144 and on 2 and 8 in fish exposed to severe hypoxia, compared with initial and control values, respectively. In fish exposed to moderate hypoxia, SSI decreased significantly on hour 24, compared with both initial and control values.

## Discussion

Pradhan *et al.*, (1989) found that erythropoiesis in fish was enhanced by human urinary EPO. In 1993, a protein, which competes with human EPO for anti-human EPO antibodies, was detected immunochemically in trout kidney, spleen and plasma and serum using a radioimmunoassay kit (EPO-Trac RIA kit; Incstar Co.Ltd, USA) which is based on  $^{125}\text{I}$ -labelled recombinant human EPO and goat anti-recombinant human EPO (Wickramasinghe, 1993). It was also reported that considerably more EPO was detected in the kidney than in the other materials

studied.

In our study an ELISA kit, based on monoclonal (murine) antibody against recombinant human EPO and rabbit polyclonal anti-recombinant human EPO, was used and immunoreactive EPO was detected in all homogenates of kidney and spleen from rainbow trout. The kidney/spleen EPO ratio in trout kept under normal DO conditions was about 8, much higher than that (about 2.5) of a previous report (Wickramasinghe, 1993). There were large annual variations in basal levels of EPO in both kidney and spleen (Kakuta *et al.*, unpublished data) and so differences in the ratio of EPO in spleen and kidney may reflect when the sample was taken. We could not detect EPO in plasma and this could be due to differences in the kits used for EPO detection (specificity of antibodies and the extent of non-specific reactions) and the methods of blood collection (utilization of heparin or EDTA as a coagulant, the extent of haemolysis). It is worthy of note, however, that in our study we were unable to detect EPO in any plasma samples, including concentrated ones. This finding indicates that in fish, EPO plays an important role in the regulation of erythropoiesis as a para-hormone, that is, renal erythropoiesis is a paracrine event controlled primarily by locally generated EPO.

In this study, renal EPO levels in rainbow trout exposed to hypoxic conditions increased significantly. The increase in renal EPO levels with hypoxia described here are the first reported observations in fish. It was also found that the increase in EPO was associated with increases in Hb concentrations and probably the number of red blood cells (RBC), as shown in mammals. On the other hand, EPO levels in the spleen were considerable lower than in the kidney, and decreased at least in the early stage of hypoxia. These findings suggest that (i) there is a similarity between fish and mammalian erythropoietic systems, (ii) the kidney is not only a main erythropoietic organ but also a major source of EPO in fish, and (iii) renal erythropoiesis in the fish is controlled primarily by locally generated EPO.

Both splenic EPO levels and splenic somatic index (SSI) decreased significantly in the early stages of hypoxic exposure. When fish are subjected to hypoxia, the spleen contracts immediately and blood cells stored in this tissue are released into blood vessels. In these experiments, decreased SSI and increased Hb concentrations in blood were observed simultaneously and presumably the increase in hemoglobin concentration was due to splenic contraction and the release of red blood cells into the circulation (Randall and Perry, 1992). EPO, however, presumably activates erythropoiesis and, although the initial rise in hemoglobin

concentration is too rapid to result from erythropoiesis, the subsequent increase in hemoglobin levels after a few days probably represents EPO driven erythropoiesis. Spleen EPO levels increased 144 and 216 hours after the exposure to moderate hypoxia. These results suggest that blood cells, in particular, ones stored in spleen, may be binding EPO, though it is possible trout spleen produces EPO and/or blood cells in the fish play an important role as a reserve stock of EPO.

The minimum oxygen level required for normal growth and reproduction is around 60 % to 70% DO saturation for rainbow trout. Trout can survive 30% DO saturation in the water, and this level represents an acute situation (severe hypoxia) for this species (Boutilier et al., 1987). Various physiological responses, such as enhancement of oxygen delivery, suppression of aerobic metabolism, and activation of anaerobic metabolism, are rapidly activated in trout exposed to severe hypoxia, Hypoxia Inducing Factor 1 (HIF-1) increases within about a few hours of hypoxic exposure in trout (Soitamo et al., 2001) and this presumably activates EPO production in fish, as in mammals. This, in turn, increases erythropoiesis, causing a rise in blood hemoglobin levels after a few days. The increase in HIF-1 was reported from a study using cultured hepatocytes. Our study was based on hypoxic exposure of intact animals and an increase in EPO was not detected until after 8 hours exposure to hypoxia. the time course of the increase in erythropoiesis, as indicated by changes in blood hemoglobin concentration, is not well established. Exposure to mild hypoxia resulted in an increase in EPO, but the increase was not significantly different from that of the same time control until after six days of hypoxic exposure. This indicates that the pattern of increase in EPO varies with the level of hypoxia. Thus hypoxic exposure results in increased levels of HIF-1 and this leads to increased EPO levels in the kidney within hours or days depending on the level of hypoxia. The time course of the increase in erythropoiesis, as indicated by changes in blood hemoglobin concentration in this study, is not well established. Erythropoiesis appears to be stimulated within a few days after the EPO increase.

There is no data on renal blood flow and kidney oxygen levels in the fish exposed to hypoxic conditions, though in mammals EPO production by the kidney is inversely related to the oxygen-delivering capacity of the blood perfusing it (Groopman et al., 1989; Spivak, 1989). Thus, although it is clear that hypoxia results in an increase in EPO levels in fish, we know nothing of the changes in blood flow and oxygen levels in the kidney of fish.

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