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EFFECTS OF ACUTE HYPOXIA ON THE ENERGY STATUS AND ANTIOXIDANT DEFENSE SYSTEM IN THE BLOOD OF CARP (*CYPRINUS CARPIO* L.)

R. V. ŽIKIĆ[1], A. Š. ŠTAJN[1],S. Z. PAVLOVIĆ[2], BRANKA I. OGNJANOVIĆ[1] SNEŽANA D. MALETIĆ[1], MIRJANA D. MARKOVIĆ [1], LJILJANA M. DRAGIĆEVIĆ-DJOKOVIĆ[3], R. M. RADOJIČIĆ[4] and ZORICA S. SAIČIĆ[2]

[1]*Institute of Biology, Faculty of Sciences, University of Kragujevac,* 34000 Kragujevac,
[2]*Institute for Biological Research "Siniša Stanković", Department of Physiology,* 11060 Belgrade,
[3]*Institute of Physiology, Faculty of Medicine, University of Kragujevac,* 34000 Kragujevac,
[4]*Institute of Physiology and Biochemistry, Faculty of Biology, University of Belgrade,* 11000 Belgrade, Serbia, Yugoslavia

Abstract - The influence of acute hypoxia on glucose, pyruvate, lipid peroxide (LP), reduced glutathione (GSH) concentrations and lactate level in the whole blood of carp (*Cyprinus carpio* L.) under aquarium conditions were studied. The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), the concentrations of ATP and ADP and ATP/ADP ratio in the red blood cells (RBCs) were analyzed. Glutathione-S-transferase (GST) activity was determined in the plasma. Our experiments showed that short-term and long-term hypoxia causes significant changes of all examined haematological parameters. Increased concentration of LP and increased SOD, CAT and GST activities, as well as a decreased GSH-Px activity showed that under hypoxic conditions oxidative stress and RBCs damage were produced.

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INTRODUCTION

In various environmental and pathological conditions, animals can be exposed to partial or complete loss of oxygen. Hypoxia is one of the most important stress factors in fish which results in changes of haematological parameters (Muusze *et al.* 1996) and many physiological and biochemical alterations (Forster *et al.* 1992; Airaksinen *et al.* 1998). Hypoxia is important in both biomedical and environmental contexts and requires a rapid adaptive response in metabolic organization (Gracey *et al.* 2001). The reduction of oxygen concentration in water increases haematocrit value, haemoglobin concentration (Muusze *et al.* 1996), as well as the number of circulating erythrocytes in the blood of fish (Soldatov 1996; Van Raaij *et al.* 1996a). A significant increase of the trout red blood cells (RBCs) count appears to be a fast adaptive response to acute hypoxia (Claireaux *et al.* 1988). During hypoxia in teleost fishes, catecholamines are released into the circulation and believed to initiate a series of physiological changes aimed at enhancing blood oxygen transport and gill oxygen diffusing capacity (Montpetit and Perry 1998).

Energy metabolism is also altered under hypoxic conditions. Exposure of teleosts to water with 33% oxygen saturation decreased liver glucose concentration (Lushchak *et al.* 1998). Van Raaij *et al.* (1996b) suggested that hypoxia-induced hyperglycaemia is likely to be the result of hepatic glycogenolysis stimulated by circulating catecholamines and a stimulation of gluconeogenesis by cortisol during recovery. The oxygen restriction resulted in a significant increase of plasma lactate level in flatfish (*Solea solea*) and carp (*Cyprinus carpio* L.) indicating anaerobic metabolism (Van den Thillart *et al.* 1994; Van Raaij *et al.* 1996b). Some authors suggested that *in vitro* exposure of trout erythrocytes to hypoxic conditions results in an enhancement of a cyclic AMP production (Reid *et al.* 1993). A significant reduction of the total pool of adenosine and guanosine phosphates was also observed in erythrocytes of rainbow trout exposed to hypoxia (Val *et al.* 1995).

Other authors showed that lipid peroxide (LP) concentration in gills and air sac biomembranes of freshwater fish was enhanced with water temperature increase inducing an increased oxidative stress (Parihar and Dubey 1996). Many investigators have found a significant changes in antioxidant defense system (AOS) in fish during acute hypoxia: superoxide dismutase (SOD) and catalase (CAT) activities were increased in the blood, liver and red muscle of teleosts, while glutathione peroxidase (GSH-Px) activity was decreased in all examined tissues, except in the blood (Wilhelm *et al.* 1993).

The aim of this study was to investigate the energy status parameters, AOS, as well as LP concentration in the blood of common carp (*Cyprinus carpio* L.) under conditions of progressive reduction of oxygen concentration. We measured the following parameters of energy status: glucose, lactate and pyruvate concentrations,

lactate/pyruvate ratio in the blood and adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) levels, as well as ATP/ADP ratio, total adenine nucleotide (TAN) and adenylate energy charge (AEC) in the red blood cells (RBCs) of fish. We also determined the activities of superoxide dismutase SOD (EC 1.15.1.1.), catalase CAT (EC 1.11.1.6) and glutathione peroxidase GSH-Px (EC 1.11.1.9) in RBCs, as well as glutathione-S-transferase GST (EC 2.5.1.18) in the plasma. The concentration of reduced glutathione (GSH) in the blood was also assessed. In addition, we evaluated lipid peroxide (LP) concentration in whole blood of animals.

Existing literature data shows only the aspects of chronic and moderate hypoxia on the AOS in different tissues of freshwater teleosts. Therefore, in these experiments we examined the influence of short-term and long-term hypoxia on the activity of antioxidant defense enzymes especially SOD, CAT and GSH-Px, as well as the changes of GSH concentration in the blood of carp (*Cyprinus carpio* L.).

MATERIALS AND METHODS

Fish

In our experiments common carps (*Cyprinus carpio* L.), weighing 430 ± 20 g were used. The animals were acclimated to the aquarium conditions with water temperature of 14.5 ± 0.5°C, pH 7.2 and concentration of dissolved oxygen of 6.5 *mg* O_2/L (100%) – control (C) in dechlorinated and aerated water for 20 days. After a period of acclimation the animals were divided in three experimental groups each consisting of 7 animals: (1) control fish – C, (2) fish exposed during 75 min to progressive O_2 decrease to 4.2 ± 0.3 *mg*/L water (65 ± 0.5%) – H1 and (3) fish exposed during 150 min of progressive O_2 decrease to 2.3 ± 0.3 *mg*/L water (35 ± 0.5%) – H2.

Measurements of oxygen concentration

The concentration of oxygen in the water was determined by using HI 9143 - Microprocessor auto cal dissolved oxygen meter (Hanna Instruments).

Preparation of the samples

After the treatment the animals were sacrificed by a sharp blow on the head always between 8 and 10 a.m. to avoid any possible rhythmic variations in metabolic and antioxidant level and fresh blood was immediately collected into heparinized test tubes.

Determination of energy status parameters

Blood extraction was done by 1.0 Vol. of ice-cold 0.6 mmol/L perchloric acid immediately after exsanguination. Perchloric acid extracts were neutralized with 0.25 Vol. of 1.0 *mmol*/L TEA – 2.3 *mmol*/L K_2CO_3 and used for enzymatic determination of glucose (Bergmeyer *et al.* 1974), lactate (Gutmann and Wahlefeld 1974), pyruvate (Jaworek *et al.* 1974) and adenine nucleotides, ATP (Lamprecht and Trautschold 1974), ADP and AMP (Jaworek *et al.* 1974). The values of these parameters were expressed in *μmol*/mL RBCs.

Measurement of antioxidant defense enzyme activities

For determination of antioxidant defense enzyme activities the blood samples were centrifuged to separate plasma and RBCs and isolated RBCs were washed three times with 2 Vol. of cold 155 *mmol*/L NaCl. Haemolysates containing about 50 *g* Hb/L were prepared according to McCord and Fridovich (1969) and used to determine CAT and GSH-Px activities. Measurement of SOD activity was conducted in the haemolysates of washed RBCs in which Hb was previously removed by the method of Tsuchihashi (1923). SOD activity in RBCs was determined by the epinephrine method (Misra and Fridovich 1972) based on the capacity of SOD to inhibit autooxidation of adrenaline to adrenochrome. One unit of SOD activity was defined as the amount of the enzyme causing 50% inhibition of the autooxidation of adrenaline at 26°C and expressed in U/*g* Hb. The Hb concentration in erythrocytes was estimated by the cyanomethaemoglobin method (Drabkin and Austin 1935). CAT activity in RBCs was assayed as described by Beutler (1982) and expressed as *μmol* H_2O_2/min/*g* Hb. The activity of GSH-Px in RBCs was evaluated by the method of (Maral *et al.* 1977) based on the measurement of nicotinamide adenine dinucleotide phosphate (NADPH) consumption and expressed as *nmol* NADPH/min/*g* Hb. GST activity was determined in the plasma according to Habig *et al.* (1974) and expressed in *nmol* GSH/min/mL plasma.

Determination of GSH and LP concentrations

The concentration of reduced glutathione (GSH) in whole blood was measured by standard method of Beutler (1975) and expressed in *nmol*/mL blood. The concentration of lipid peroxide (LP) in the blood was determined as thiobarbituric acid-reactive substances (TBARS) according to Ohkawa *et al.* (1979) and expressed in *nmol*/mL blood.

Chemicals

All chemicals used in our experiments were Sigma (St. Louis, MO, USA) products.

Statistical analysis

Data are given as means ± SE. All obtained results were compared with those of the control animals (C). Statistical analysis of the results was based on the Student's paired t-test considering the significance at a level of $p < 0.05$ (Hoel 1966).

RESULTS

Changes in energy status parameters during acute hypoxia

The results presented in this study demonstrate that acute hypoxia induced significant changes in glycolytic parameters in the blood of carp. Table 1. shows a significant decrease of glucose ($p<0.01$) in H1 - group and pyruvate concentrations in both, H1 and H2 group of fish ($p<0.005$), while lactate concentration was significantly increased ($p<0.05$) only in H2 group. According to these data lactate/pyruvate ratio in H1 and H2 experimental group was 2.38- ($p<0.05$) and 3.77- fold ($p<0.01$) higher, respectively (Table 1).

Table 1. The effects of acute hypoxia on glucose concentration in the whole blood and energy status parameters in the red blood cells (RBCs) of control carps (C, 100% of oxygen), carps exposed to 65 ± 0.5% of oxygen during 75 min (H1) and those exposed to 35 ± 0.5% of oxygen during 150 min (H2).

	C	H1	H2
Blood glucose (*μmol*/mL RBs)	5.07 ± 0.30	3.46 ± 0.14***	4.15 ± 0.28
Lactate (*μmol*/mL RBs	1.02 ± 0.16	1.04 ± 0.13	1.65 ± 0.15*
Pyruvate (*μmol*/mL RBs	0.021 ± 0.004	0.009 ± .002****	0.009±0.003****
Lac/pyr	48.57 ± 5.00	115.55 ± 11.55*	183.30±18.33***
ATP (*μmol*/mL RBs	7.65 ± 0.20	6.87 ± 0.18*	6.14 ± 0.11***
ADP (*μmol*/mL RBs	0.29 ± 0.02	0.30 ± 0.03	0.35 ± 0.005*
AMP (*μmol*/mL RBs	0.29 ± 0.02	0.30 ± 0.03	0.057 ± 0.003
[a]TAN (*μmol*/mL RBs	8.01 ± 0.22	7.20 ± 0.15*	6.53 ± 0.11****
ATP/ADP	26.71 ± 1.67	25.88 ± 2.82	17.63 ± 0.50***
[b]AEC	0.97 ± 0.002	0.097 ± 0.003	0.96 ± 0.002

[a]TAN = ATP + ADP +AMP; [b]AEC = (ATP + 1/2 ADP)/TAN
The values are means ± SE from seven animals in each group.
*p<0.05; ***p<0.01; ****p<0.005

The energy status of RBCs in acute hypoxia of both groups of treated carps was significantly altered. The concentration of ATP was significantly decreased in both, H1 and H2 group ($p<0.05$, $p<0.01$, respectively), while ADP level was significantly increased only in the H2 - group of animals ($p<0.05$) (Table 1). The AMP level was not significantly changed in any of investigated groups (Table 1). Total adenine nucleotide level (TAN) was significantly decreased in both experimental groups of carps ($p<0.05$, $p<0.005$, respectively).

On the basis of the given parameters of energy status, ATP/ADP ratio and adenylate energy charge (AEC-an additional index of cell energy status) were calculated. Owing to the disproportionate decrease of ATP the ATP/ADP ratio was significantly lower only in H2 - group of carps ($p<0.01$). On the other hand, AEC was not altered in the blood of fish after exposure to both treatments (H1 and H2) in comparison with the controls.

Effects of acute hypoxia on antioxidant defense enzyme activities

The data presented in Figs. 1. and 2. shows that acute hypoxia induced significant changes in the antioxidant defense enzyme activities. In carp exposed to acute hypoxia the activities of SOD and CAT (Fig. 1) were significantly increased only in the H1 group ($p<0.05$ and $p<0.01$, respectively), while GST activity (Fig. 2) was significantly increased in both, H1 and H2, groups of animals ($p<0.05$, $p<0.01$, respectively). However, GSH-Px activity was significantly decreased (Fig. 2) in the fish exposed to acute hypoxia (H1 and H2) as compared to the controls ($p<0.005$).

Effects of acute hypoxia on GSH and LP concentrations

The observed decrease in blood GSH concentration in both H1 and H2 group of fish was not statistically significant (Table 2). At the same time, the concentration of LP (Table 2) was significantly increased in the blood of carps of both those groups in comparison with the controls ($p<0.05$).

DISCUSSION

Hypoxia induces many harmful effects in the teleosts and alters haematological (Claireaux *et al.* 1988; Muusze *et al.* 1996; Soldatov 1996; Van Raaij *et al.* 1996a), biochemical and physiological parameters (Reid *et al.* 1993; Van den Thillart *et al.* 1994; Val *et al.* 1995; Van Raaij *et al.* 1996a,b; Lushchak *et al.* 1998).

Table 2. The effects of acute hypoxia on the concentration of reduced glutathione (GSH, nmol/mL blood) and on the lipid peroxide (LP, nmol/mL blood) concentration in the blood of control carps (C, 100%), carps exposed to 65 ± 0.5% of oxygen during 75 min (H1) and carps exposed to 35 ± 0.5% of oxygen during 150 min (H2).

	C	H1	H2
GSH (*nmol*/ml blood)	171.0 ± 21.4	154.6 ± 9.6	142.1 ± 14.2
LP (*nmol*/ml blood)	1.99 ± 0.07	2.73 ± 0.30*	2.31 ± 0.80*

The values are means ± SE from seven animals in each group. *$p<0.05$.

As shown in Table 1. a progressive reduction of oxygen concentration induced a decrease in glucose (H1) and pyruvate (H1 and H2) concentrations, while lactate concentration was significantly increased (H2), indicating a stimulation of anaerobic glycolysis. These results resemble those of numerous authors (Pesquero *et al.* 1994; Van den Thillart *et al.* 1994; Via *et al.* 1994; Van Raaij *et al.* 1996b; Lushchak *et al.* 1998). A significant increase of lactate and a decrease of pyruvate concentration were accompanied by an increased lactate/pyruvate ratio (Table 1). Hypoxia-induced increase of lactate/pyruvate ratio, a representative NAD/NADH ratio, suggests oxidoreductive instability in carp RBCs.

In RBCs of carp energy is provided by glycolysis in the cytoplasm, as well as by oxidative phosphorylation in mitochondria. Hence, hypoxia-induced inhibition of oxidative phosphorylation through Pasteur effect (Rapoport 1986) was followed by the stimulation of glycolysis. The same mechanism of glycolysis stimulation was apparent in reticulocytes (the RBCs with functional mitochondria) of anemic Belgrade laboratory rats, where intracellular iron deficiency-induced hypoxia inhibited oxidative phosphorylation (Maletić *et al.* 2000). A strong inhibition of respiration by nitric oxide donors was also followed by 2.4- fold stimulation of glycolysis in rat reticulocytes (Maletić *et al.* 1999a,b). Alternative explanation for increased rate of glycolysis in RBCs of carps may involve hypoxia-induced stimulation of glycolytic enzymes(Lushchak *et al.* 1998).

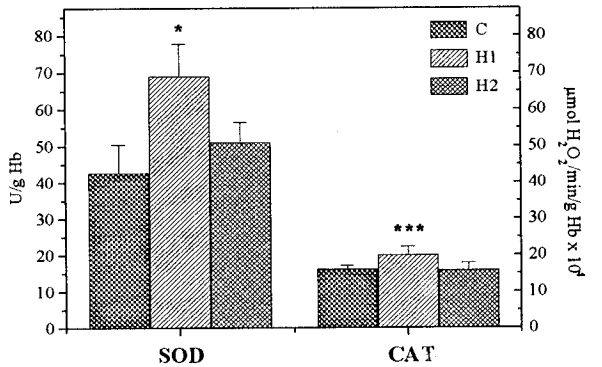
The results of our study show a significantly lower ATP level that is in accordance with previous data (Via *et al.* 1994; Val *et al.* 1995), as well as concomitant increase of ADP concentration in H2 group exposed to prolonged hypoxia. This data indicates that hypoxia-induced stimulation of glycolytic ATP production is not sufficient to compensate for decreased energy production due to the inhibition of oxidative phosphorylation. Considering this result, a decline of ATP concentration, as well as an increase in ADP levels during hypoxia may be important since ATP inhibits, while ADP and AMP stimulate the activity of phosphofructokinase, which is the main regulatory enzyme of the glycolytic metabolic pathway (Rapoport 1986; Maletić *et al.* 1999a,b).

Owing to a disproportionate decrease of ATP levels, decreased ATP/ADP ratio (Table 1) suggests a stimulation of energy-consuming processes and probably increased catecholamine levels during deep hypoxia (Perry and Reid 1994; Van Raaij *et al.* 1996a). Namely, *in vitro* exposure of trout erythrocytes to hypoxic conditions results (Reid *et al.* 1993; Gilmour *et al.* 1994) through the enhancement of erythrocyte cyclic AMP, in increased responsiveness of the erythrocyte Na^+/H^+ antiporter (an ATP-consuming system) to catecholamines. Roig *et al.* (1997) suggested that under the circumstances when the respiratory chain was blocked, adrenergic stimulation increased the rates of ATP consumption and glycolysis of RBCs. At the same time, adjustments of intraerythrocytic ATP and GTP significantly improved oxygen transfer in fish during environmental hypoxia (Val 1995).

The results presented here reveal that in hypoxic carps there are marked changes in the RBC antioxidant status. The activities of SOD and CAT were significantly increased in RBCs of H1 group of carp indicating a fast adaptive response of these enzymes to the hypoxic conditions. However, after prolonged hypoxia followed by oxygen reduction to 2.3 ± 0.3 *mg*/L in H2 group the activities of SOD and CAT were normalized and returned to control values (Fig. 1). This suggests that after prolonged exposure to hypoxia (H2), carp RBCs can balance production of reactive oxygen species and activities of SOD and CAT. Lemaire *et al.* (1993) showed that partial reduction of molecular oxygen produces reactive oxygen species, including the superoxide anion radical and hydroxyl radical in all fish tissues. Therefore, it is reasonable to expect an increased activity of SOD in RBCs of hypoxic carps. Similar results were obtained in other vertebrate species that were submitted to other environmental stress factors (Žikić *et al.* 1998). The activity of CAT was also increased in RBCs of hypoxic carps (Fig. 1). Wilhelm *et al.* (1993) showed that acute hypoxia leads to an increased CAT activity in all tissues of teleosts. Therefore, the increased activity of CAT may be a consequence of more intensive pro-

duction of H_2O_2 through the mechanism of dismutation of superoxide anion radicals (Petrović *et al.* 1983; Saičić *et al.* 1991,1997; Halliwell and Gutteridge 1999; Žikić *et al.* 2000). Other authors (Lushchak *et al.* 2001) demonstrated that acute anoxia induced a significant increase of liver CAT activity in the goldfish *(Carassius auratus)*. According to our data (Fig. 2), the activity of GST was significantly increased in the plasma of carp exposed to acute hypoxia and may be of importance to minimize the damage during reintroduction of oxygen (Storey 1996). Furthermore, increased activity of GST was also found in the plasma of rats exposed to heavy metals, such as cadmium and plays an important role in prevention of oxidative stress (Kostić *et al.* 1993).

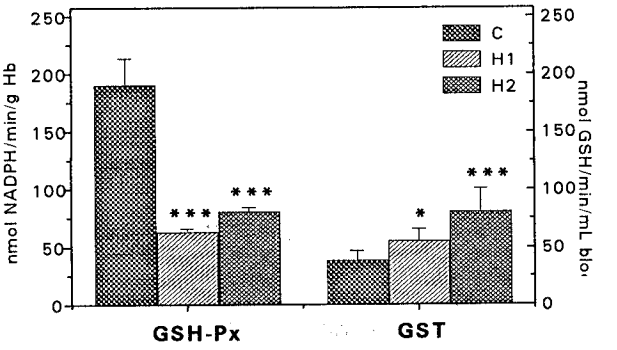
Fig. 1. The effects of acute hypoxia on the activities of superoxide dismutase (SOD, U/g Hb) and catalase (CAT, μmol H_2O_2/min/g Hb) in RBCs of control carps (C, 100%), carps exposed to 65 ± 0.5% (H1) and 35 ± 0.5% (H2) of oxygen.The values are means ± SE from seven animals in each group. The results were compared with those of the control animals (C).
*p<0.05; ***p<0.01.



Decreased activity of GSH-Px (Fig. 2) developed during acute hypoxia indicates that this system may not participate in protection of RBCs of carps against acute hypoxia. Other investigators demonstrated either a decreased (Wilhelm *et al.* 1993) or increased (Lushchak *et al.* 2001) activity of GSH-Px in some tissues of various fish species under hypoxic conditions depending on the duration of asphyxial conditions and on the fish species studied. Owing to increased activity of CAT in RBCs of carp, we suppose that CAT plays a more important role in detoxification of hydrogen peroxide in RBCs than GSH-Px.

Fish exposed to a lower concentration of dissolved oxygen (H2) showed significant changes in antioxidant status of RBCs and a certain adaptive response of SOD, CAT and GSH-Px activities. It is well known that extracellular GSH plays an important role in protection of proteins against thiol oxidation during the phase of hypoxia (Seiler and Starnes 2000).

Fig. 2. The effects of acute hypoxia on the activities of glutathione peroxidase (GSH-Px, nmol NADPH/min/g Hb) in RBCs and glutathione-S-transferase (GST, nmol GSH/min/mL blood) in the plasma of control carps (C), carps exposed to 65 ± 0.5% (H1) and 35± 0.5% (H2) of oxygen. The values are means ± SE from seven animals in each group. The results were compared with those of the control animals (C).
*p<0.05; ***p<0.01; ****p<0.005.



In other tissues, such as brain it was established that hypoxia induced a significant depletion of GSH, accompanied by an increased concentrations of oxidized glutathione (GSSG) and LP (Barth *et al.* 1998). The obtained data suggests that decreased glucose availability (Table 1) for NADPH production during hypoxia impairs GSSG reduction, compromises hydroperoxide metabolism and increases peroxide output (LeGrand and Aw 1998).

The data obtained in our study shows that acute hypoxia significantly increases LP in the blood of both experimental groups in comparison with the controls (Table 2). Similar results were obtained by Gus'kov *et al.* (1999) who used amphibian *Xenopus laevis* as a model. Rana and Singh (1996) and Pavlović *et al.* (2001) showed that extreme conditions, such as cadmium toxicity or other environmental stress factors induce LP production in the blood and tissues of freshwater teleosts, as well as in rats. Increase of the temperature (and following loss of oxygen) also leads to an increased concentration of LP in gills and air sac, indicating an increased oxidative stress (Parihar and Dubey 1996). Investigations on other vertebrates also showed increased lipid peroxidation and confirmed an oxidative damage of tissues as well (Žikić *et al.* 1998; Pavlović *et al.* 2000).

In conclusion, acute hypoxia induced significant changes in energy status, components of antioxidant defense system and lipid peroxide concentration in the blood of carp (*Cyprinus carpio* L.) under the of progressive reduction of oxygen concentration. All specified changes are dependent on the duration of

hypoxic conditions. The present results suggest that during the evolution carps developed a well defined metabolic regulation and antioxidant defense system for the protection against acute hypoxia.

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ЕФЕКТИ АКУТНЕ ХИПОКСИЈЕ НА ЕНЕРГЕТСКИ СТАТУС И АНТИОКСИДАЦИОНИ ЗАШТИТНИ СИСТЕМ У КРВИ ШАРАНА (*CYPRINUS CARPIO* L.)

Р. В. ЖИКИЋ[1], А. Ш. ШТАЈН[1], С. З. ПАВЛОВИЋ[2], БРАНКА И. ОГЊАНОВИЋ[1], СНЕЖАНА Д. МАЛЕТИЋ[1], МИРЈАНА Д. МАРКОВИЋ[1], ЉИЉАНА М. ДРАГИЋЕВИЋ-ЂОКОВИЋ[3], Р. М. РАДОЈИЧИЋ[4] и ЗОРИЦА С. САИЧИЋ[2]

[1]*Институт за биологију, Природно-математички факултет, Универзитет у Крагујевцу,* 34000 Крагујевац,
[2]*Институт за биолошка истраживања "Синиша Станковић", Одељење за физиологију,* 11060 Београд,
[3]*Институт за физиологију, Медицински факултет, Универзитет у Крагујевцу,* 34000 Крагујевац,
[4]*Институт за физиологију и биохемију, Биолошки факултет, Универзитет у Београду,* 11000 Београд, Србија, Југославија

Утицај акутне хипоксије на концентрацију глукозе, пирувата, липидних пероксида (LP), редукованог глутатиона (GSH) и на ниво лактата у крви шарана (*Cyprinus carpio* L.) је анализиран у акваријумским условима. У црвеним крвним зрнцима (RBCs) је анализирана активност ензима супероксид-дисмутазе (SOD), каталазе (CAT), глутатион-пероксидазе (GSH-Px) као и концентрације ATP, ADP и однос ATP/ADP. У плазми је анализирана активност глутатион-S-трансферазе (GST). У нашим експериментима је утврђено да краткотрајна прогресивна хипоксија изазива значајне промене свих испитиваних хематолошких параметара. Повећање концентрације LP и повећање активности ензима SOD, CAT и GST уз истовремено смањење активности GSH-Px потврђују настанак оксидационог стреса и оштећење RBCs под хипоксичним условима.