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Metabolic responses to prolonged starvation, food restriction, and refeeding in the brown trout, *Salmo trutta*: Oxidative stress and antioxidant defenses

Abdulkadir Bayir *, A. Necdet Sirkecioglu, Mehtap Bayir, H. Ibrahim Haliloglu, E. Mahmut Kocaman, N. Mevlut Aras

Department of Fisheries and Aquaculture Engineering, Faculty of Agriculture, University of Atatürk, 25240, Erzurum, Turkey

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ABSTRACT

The effects of long-term starvation and food restriction (49 days), followed by refeeding (21 days) have been studied with respect to antioxidant defense in the liver and gills (branchial tissues) of the brown trout, *Salmo trutta*. Malondialdehyde levels in both tissues increased in parallel with starvation and food restriction and these values did not return to normal after the refeeding period. The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) in liver and gills increased during the 49 days of starvation, but glucose-6-phosphate dehydrogenase (G6PD) activities decreased. Glutathione *S*-transferase (GST) activity decreased in the liver at the 49th day of starvation, but increased in the branchial tissues. Some of the antioxidant enzyme activities (such as hepatic GST and branchial G6PD) returned to control values of fed fish after the refeeding period, but others (e.g. hepatic SOD and branchial GPx) did not return to normal values. In conclusion, our study indicates that total or partial food deprivation induces oxidative stress in brown trout.

1. Introduction

Reactive oxygen species (ROS) are free radicals and/or oxygen derivatives that are continuously generated as products of oxidative metabolism under physiological conditions (Winston and Di Giulio, 1991; Matés, 2000). Oxidative stress occurs when this ROS generation exceeds its removal (Sies, 1986). Oxidative stress is widely thought to lead to damage of DNA, proteins, steroid components, and unsaturated lipids in cell membranes (Halliwell and Gutteridge, 2000; Martínez-Álvarez et al., 2005). Fish tissues contain large quantities of unsaturated lipids (polyunsaturated fatty acids, PUFAs), which are essential for membrane function. A large PUFA content implies an elevated risk of oxidative stress, since these lipids are major targets for ROS (Abele and Puntarulo, 2004; Martínez-Álvarez et al., 2005). Therefore, lipid peroxidation (LPOX) is a useful biochemical indicator of oxidative damage (van der Oost et al., 2003).

Fish, like other aerobic organisms, have a variety of enzymatic and non-enzymatic antioxidant scavenging systems that maintain endogenous ROS at relatively low levels and attenuate the damage related to the high reactivity of ROS (Wilhelm Filho et al., 2001). The key antioxidant players in this antioxidant defense system include superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.16), glutathione peroxidase (GPx, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2), glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) and glutathione *S*-transferase (GST, EC 1.5.1.18) (Halliwell and Gutteridge, 2000; Aras et al., 2009). In fish, these antioxidant defenses can be influenced both by intrinsic factors such as systematic position, age, feeding behavior, food consumption, and diet type and also by extrinsic factors such as toxins present in the water, seasonal and daily changes in dissolved oxygen, and water temperature (Martínez-Álvarez et al., 2005; Cunha Bastos et al., 2007; Aras et al., 2009; Solé et al., 2009).

In their natural habitats, fish often experience periods of poor food supply in response to several factors (e.g., temperature, spawning migration, reproduction, etc.) and they are well adapted to long-term starvation (Van Dijk et al., 2005; Pérez-Jiménez et al., 2007; Furné et al., 2009). Cultured fish also experience similar situations derived from these same factors as well as from factors arising from routine aquaculture procedures (Pérez-Jiménez et al., 2007). Previous studies have reported that antioxidant defenses in fish are activated by starvation (Pascual et al., 2003; Morales et al., 2004; Pérez-Jiménez et al., 2007; Furné et al., 2009). However; to date, no information has been reported regarding the antioxidant defenses in relation to starvation, food restriction, and refeeding in the brown trout, *Salmo trutta*.

Trout are the most valuable group of fish species in the world; and low prices for salmonids over the past years appear to be due chiefly to the large production of salmonids by aquaculture (Rasmussen and Ostenfeld, 2000; Kocaman et al., 2009). Although the rainbow trout, *Oncorhynchus mykiss*, is the principal cultured salmonid in Turkey,

* Corresponding author at: Department of Fisheries and Aquaculture Engineering, Faculty of Agriculture, University of Atatürk, 25240, Erzurum, Turkey. Tel.: +90 442 2311079; fax: +90 442 2360958.
E-mail address: abayir@atauni.edu.tr (A. Bayir).

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consumers prefer brown trout to rainbow trout for its taste, color, and nutritional value. Therefore, its price is typically twice that of rainbow trout (Kocaman et al., 2006; Kaya and Erdem, 2009).

Most of the research on oxidative stress studies in fish has focused on toxicological aspects, such as the effects of xenobiotics and heavy metals on the antioxidant defense system (Lemaire and Livingstone, 1993; Rodriguez-Ariza et al., 1993; Vaglio and Landriscina, 1999; Lopes et al., 2001; Li et al., 2003). Other studies have investigated the effects of season or other stressors on the activities of antioxidant enzymes (Solé et al., 2006; Bagnyukova et al., 2007; Özmen et al., 2007; Aleshko and Lukyanova, 2008; Aras et al., 2009; Bayır et al., 2011). The present study was undertaken to investigate the effects of long-term starvation, food restriction and refeeding on the antioxidant defenses in the liver and gills of the brown trout.

2. Materials and methods

2.1. Fish and experiment design

Brown trout (*Salmo trutta*, 179.70 ± 7.97 g body mass) were obtained from the Trout Research and Extension Center of the Agriculture Faculty at the Atatürk University. Fish were distributed randomly in four 2000-L circular tanks for a total of 120 fish $tank^{-1}$. One replicate per treatment is used owing to limited place in experimental unit. The tanks were supplied with 9–10 °C well water at a flow rate of 0.75 L s^{-1}. A diurnal light: dark cycle of 12:12 h was provided by fluorescent lighting. Fish were acclimated to the experimental conditions for 2 weeks and fed to satiety twice a day with a commercial trout feed (45% crude protein, 20% crude lipid, 10% crude ash, and 3% crude carbohydrates; Sibal Feed, Sinop, Turkey). Following the acclimation period, three treatment groups were fed different amounts of food, at 1, 0.5, and 0% body mass day^{-1} three times daily, at 08:00, 12:00, and 16:00 h, for 49 days. The fish in the control group were hand-fed to apparent satiety three times daily. Seven fish of each treatment were captured at 7, 14, 21, 28, 35, 42, and 49 days. After the starvation and food restriction period, all of the remaining fish were hand-fed to apparent satiety for a 21 day period (days 49 to 70), and sampled at days 56, 63, and 70 for the refeeding treatment. At the sampling days, captured fish were killed by a sharp blow to the head. Fish were weighed individually, and then livers and gills were excised and frozen immediately in liquid nitrogen. Samples were transferred to the laboratory and stored at −84 °C. Hepatosomatic indexes (HSI) of fish were determined as liver mass/fish mass * 100.

2.2. Determination of enzyme activities and lipid peroxidation levels

Livers and gills of fish were washed in ice-cold 0.9% NaCl, dried with filter paper. Samples (*c.* 1 g) were homogenized in 10 vol (w/v) ice-cold 100 mM Tris–HCl buffer containing 0.1 mM EDTA and 0.1% triton X-100 (v/v), pH 7.8 and a protease inhibitor cocktail (Sigma P-2714). All processes were performed on ice. Homogenates were centrifuged at 11312 *g* for 30 min at 4 °C and the resulting supernatants were used for analysis.

The following enzyme activities were measured using a double beam spectrophotometer (Thermo, Evolation 100, England):

Superoxide dismutase activity was assayed by inhibition of xanthine/xanthine oxidase generated $O_2^{\cdot-}$ reduction of nitroblue tetrazolium (NBT) (Sun et al., 1988). NBT was reduced to blue formazon by $O_2^{\cdot-}$ which has a strong absorbance at 560 nm. One unit was defined as the amount of protein producing 50% inhibition of reaction. The reaction mixture contained 100 mM Tris–HCl, 5 mM EDTA buffer (pH 8.0), 150 μM NBT, 400 mM Na_2CO_3, 1 g L^{-1} bovine serum albumin, 3 mM L^{-1} xanthine, 0.833 U mL^{-1} xanthine oxidase, 0.8 mM $CuCl_2$ and tissue homogenate (30 μL).

Glutathione peroxidase activity was determined by following the rate of nicotinamide adenine dinucleotide phosphate (NADPH) oxidation at 340 nm by the coupled reaction with glutathione reductase (GR) (Beutler, 1975; 1984). The assay system contained 100 mM Tris–HCl, 5 mM EDTA (pH 8.0), 0.1 M GSH, 10 U mL^{-1} GR, 2 mM NADPH, 7 mM t-butylhydroperoxide, distilled water and tissue homogenate (10 μL).

Catalase (CAT) activity was measured according to the method of Beutler (1984) following the decomposition of H_2O_2 at 230 nm ($\varepsilon = 0.071\ mM^{-1}\ cm^{-1}$). The assay mixture consisted of 50 mM phosphate buffer (pH 7.0), 19 mM H_2O_2 and tissue homogenate (10 μL).

Glutathione reductase activity was determined as described by Beutler (1984) by measuring the oxidation of NADPH at 340 nm using the extinction coefficient 6.22 $mM^{-1}cm^{-1}$. The reaction mixture contained 100 mM Tris–HCl, 5 mM EDTA (pH 8.0), 33 mM GSSG, 2 mM NADPH, distillated water and tissue homogenate (10 μL).

Glucose-6-phosphate dehydrogenase activity was assayed as described by the method of Beutler (1984). The activity assay was done by monitoring the increase in absorption at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) due to the reduction of 2 mM $NADP^+$. The assay system contained 100 mM Tris–HCl, 5 mM EDTA (pH 8.0), 100 mM $MgCl_2$, 2 mM $NADP^+$, 6 mM G6P, distilled water and tissue homogenate (50 μL).

Glutathione *S*-transferase activity was evaluated with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate in a final reaction mixture containing 110 mM phosphate buffer (pH 6.5), 30 mM CDNB, 100 mM GSH, distilled water and tissue homogenate (50 μL) (Habig et al., 1974). The activity rate was measured as change in optical density at 340 nm ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$).

The level of LPO was determined by assessment of malondialdehyde (MDA) as total thiobarbituric acid (TBARS)-reactive products by method of Uchiyama and Mihara (1978). The breakdown product of 1,1,3,3-tetraethoxypropane was used as a standard. Tissue homogenates were mixed with phosphoric acid (1% v/v) and TBA (0.6% w/v); the mixture was mixed strongly and incubated in a boiling water bath for 45 min. After cooling the tubes, *n*-butanol was added and mixed and the precipitates were removed by centrifugation at 4200 *g* for 10 min. Finally, the MDA concentration in the supernatant fraction was determined spectrophotometrically at 532 nm.

Specific enzyme activities were defined as enzyme unit (EU) per mg protein and MDA concentrations were expressed as nmol per mg protein. All chemicals were obtained from Sigma-Aldrich (USA).

2.3. Protein content of samples

The protein content of samples was determined by Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

2.4. Data analyses

The statistical analyses were performed with SPSS version 10.0 for Windows (SPSS, 1996). Data were presented as mean ± standard deviation (SD) of the mean. Data were analyzed by one-way analysis of variance (ANOVA) and significant differences were determined by Duncan's multiple range post-hoc test ($P<0.05$; $n = 7$).

3. Results

3.1. Body mass and hepatosomatic index

Changes in the body mass and HSI induced by long-term starvation, food restriction, and refeeding in brown trout are shown in Table 1. Starved fish lost 17.3% their initial body mass by the end of 49 days of starvation. Similarly, starvation and food restriction