Effects of water reuse system on antioxidant enzymes of rainbow trout (*Oncorhynchus mykiss* W., 1792)

I. Ozmen[1], A. Bayir[2], M. Cengiz[2], A. N. Sirkecioglu[2], M. Atamanalp[2]

[1]Biotechnology Application and Research Center, [2] Department of Fisheries, Faculty of Agriculture, Ataturk University, Erzurum, Turkey

ABSTRACT: The objective of the present study was to investigate the importance of the antioxidant enzymes; glucose-6-phosphate dehydrogenase (G6PD), glutathione reductase (GR), superoxide dismutase (SOD), glutathione peroxidase (GP) and catalase (CAT) in defense against toxic reactive oxygen species (ROS). For this purpose, comparative studies were performed on the antioxidant enzyme activities in red cells of rainbow trout (*Oncorhynchus mykiss*) between flow-through and recirculated breeding systems G6PD, GR and SOD activities were found significantly lower in reuse system than flow-through system. But, GP and CAT activities were increased significantly in the erythrocyte of rainbow trout held in reuse water tanks.

Keywords: recirculating systems; rainbow trout; reuse water; stress; antioxidant enzymes

Three different methods have been used for aquacultural production; pond culture, flow-through and recirculating systems. Pond systems are the most widely practiced form of aquaculture in the world. Recirculating systems are semi-closed systems, in which water flowing through a series of tanks or raceways is captured, treated and reused (Adler et al., 2000). Flow-through systems are traditional production methods used in farms which have high and economic water inputs. These systems discharge water from ponds to environment without any process (Aras et al., 2000).

Recirculating aquaculture systems are a type of flowing water (intensive) fish culture technology in which a high percentage of the water is reused after treatment (Summerfelt, 1996). These systems use the least amount of water, which is an advantage in areas with either limited water resources or stringent discharge standards (Adler et al., 2000). Recirculating systems have higher capital and operating costs than traditional production technologies. So, these systems are being used more often to produce in limited water farms and for expensive fish species (Noble and Summerfelt, 1996).

Reactive oxygen species (ROS) and other pro-oxidants are continually detoxified and removed in cells by antioxidant defence systems comprising both antioxidant enzymes and small molecular weight free radical scavengers. Exposure of organisms to pro-oxidant attack can increase antioxidant defences, for example, by increasing synthesis of antioxidant enzymes (Halliwell and Gutteridge, 1999). If antioxidant defences are effective in detoxifying ROS, then no harmful consequence results to the tissues. However, if the ROS attack is severe, then antioxidant defence systems may be overwhelmed, resulting in inhibition of antioxidant enzymes and oxidative damage to lipid, protein, DNA and other key molecules. Such processes may in turn provoke alterations in molecular and membrane structures and functions leading to cell and tissue damage (Reznick et al., 1998). Increased concentrations of lipid peroxidation end-products and DNA damage have been used as indicators of ROS-derived damage in biological systems (Halliwell and Chirico, 1993). These enzymatic mechanisms associated with non enzymatic systems can prevent the formation of oxyradicals or intercept oxidative propagation

reactions promoted by the oxyradicals once formed (Porte et al., 1991).

This study was undertaken to determine the effects of recirculating water systems on antioxidant enzyme activities of rainbow trout (*Oncorhynchus mykiss*). It has been investigated whether recirculating systems lead to increase free radicals by analyzing the activities of antioxidant enzymes used as complementary parameters in environmental monitoring.

MATERIAL AND METHODS

Fish source and maintenance

Rainbow trout were obtained from Trout Research and Extension Center of Ataturk University Agricultural Faculty. Fish were held in 600 l water volume fiberglass tanks (2 tanks for control, 2 tanks for recirculating systems). Fish were settled as 5 fish per tank (600 l) under natural light conditions, two tanks with a constant flow (1.5 l/min) of aerated dechlorinated tap water at 11.5 ± 1°C. The water of other tanks was recirculated with Eheim Large Canister Filters (EHEIM 2260). For controlling water temperatures of recirculated tanks, ice cubes, made with dechlorinated water, added to tanks daily. The treatment lasted 6 weeks and fish were fed twice a day with commercial trout feed during the experiments. Fish (n = 20) used in the experiment were weighted as 145 ± 14 g and the temperature of water during the experiment was measured as 13.5 ± 1.0°C.

Approximately 3 cm^3 blood samples were drawn from cardiac puncture with heparinized syringes. It was immediately centrifuged under refrigeration at 3 000 g for 10 minutes. Plasma and buffy coat were carefully removed and the separated cells washed thrice with cold saline phosphate buffer, pH 7.4 (sodium phosphate buffer containing 0.15 mol/l NaCl) and the pellet was hemolysed with five volumes of ice-cold water. The hemoglobin (Hb) concentration was determined in the hemolysates (van Kampen and Zijlstra, 1961).

Enzyme assays

G6PD activity was measured spectrophotometrically as described by Beutler (Beutler, 1984). The activity measurement was done by monitoring the increase in absorption at 340 nm due to the reduction of $NADP^+$ at 37°C. One enzyme unit represents the reduction of 1 μmol of $NADP^+$/min at pH 8.0.

GR was determined by reduced NADPH and GSSG substrates. One unit of GR was defined as the quantity of NADPH consumed per minute, which catalyzed the reduction of 1 mM of GSSG (Beutler, 1984).

SOD activity was assayed using the nitroblue tetrazolium (NBT) method of Sun (Sun et al., 1998). NBT was reduced to blue formazan by O_2^- which has a strong absorbance at 560 nm.

Total GP activities were determined as described by Floche and Gunzler in which the reduction of the product GSSG was linked to the oxidation of NADPH at 340 nm using added GR (Floche and Gunzler, 1984).

CAT activity was measured by the method of Claiborne (10) as described by Beers and Sizer (1952). Briefly, the assay mixture consisted of 0.05 M phosphate buffer saline (PBS) (pH = 7), H_2O_2 (0.019 M) and sample. Change in absorbance was recorded following the reduction of hydrogen peroxide at 240 nm during 1 min (Beers and Sizer, 1952). The red cells were haemolysed in 9 volumes of 20 mM phosphate buffer pH 7.4, the Haemolysate was used directly for enzyme assays.

Statistical analyses

All statistical analysis was performed using Statistica software release 6.0. Analysis of variance was performed and means were separated using Fisher's protected least significant differences (LSD) test at $P \leq 0.001$, $P \leq 0.01$ and $P \leq 0.05$.

RESULTS AND DISCUSSION

This is first study concerning with antioxidant defense system in the fish resident in reuse water. Activities of antioxidant enzymes and the levels of free radical scavengers have been found to correlate with various physiological or pathological conditions, including stress (Czene et al., 1997; Wojtaszek, 1997) (Figure 1). It is well known that stress leads to a series of biochemical, physiological and behavioral changes, thus, altering normal body homeostasis (Da Silva et al., 1998). SOD is the antioxidant enzyme catalyzing the dismutation of the highly reactive superoxide anion to O_2 and to the less reactive species

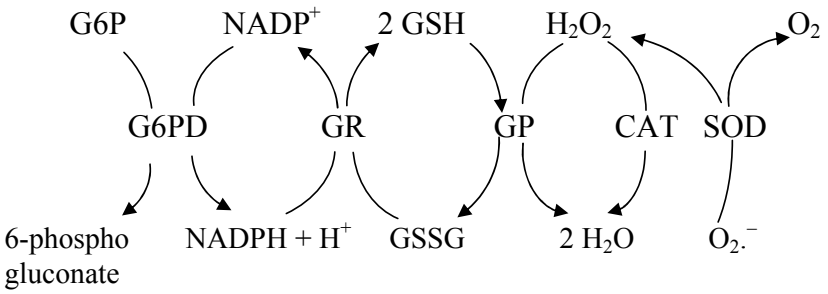


Figure 1. The catalyzing reactions by G6PD, GR, SOD, GP and CAT. H_2O_2 can be detoxified by CAT and GP, the latter of which is dependent upon GSH maintained by NADPH-dependent reduction of glutathione disulfide (GSSG) by GR. NADPH levels in turn are maintained by G6PD. SOD catalyzes to scavenge excess superoxide anions and convert them to H_2O_2

H_2O_2 (Teixeria et al., 1998). CAT reacts very efficiently with H_2O_2 to form water and molecular oxygen; and with H donors (methanol, ethanol, formic acid, or phenols) with peroxidase activity. GP catalyses the reduction of hydroperoxides using GSH, thereby protecting cells against oxidative damage. GSSG is reduced to GSH by GR. This reaction enzymatically requires NADPH produced by G6PD and 6-phosphogluconate dehydrogenase in the pentose phosphate pathway (Urso and Clarkson, 2003)

In this study, antioxidant enzyme activities involved in antioxidant defense mechanisms were investigated in the red cells of rainbow trout. The results of enzyme activities of flow-through and reuse water group were shown in Table 1 and Figure 2. The data from this study indicate that the fish resident in reuse water may be exposed to oxidative stress in the erythrocytes. Comparison between flow-through and reuse water group revealed differences in enzyme activities.

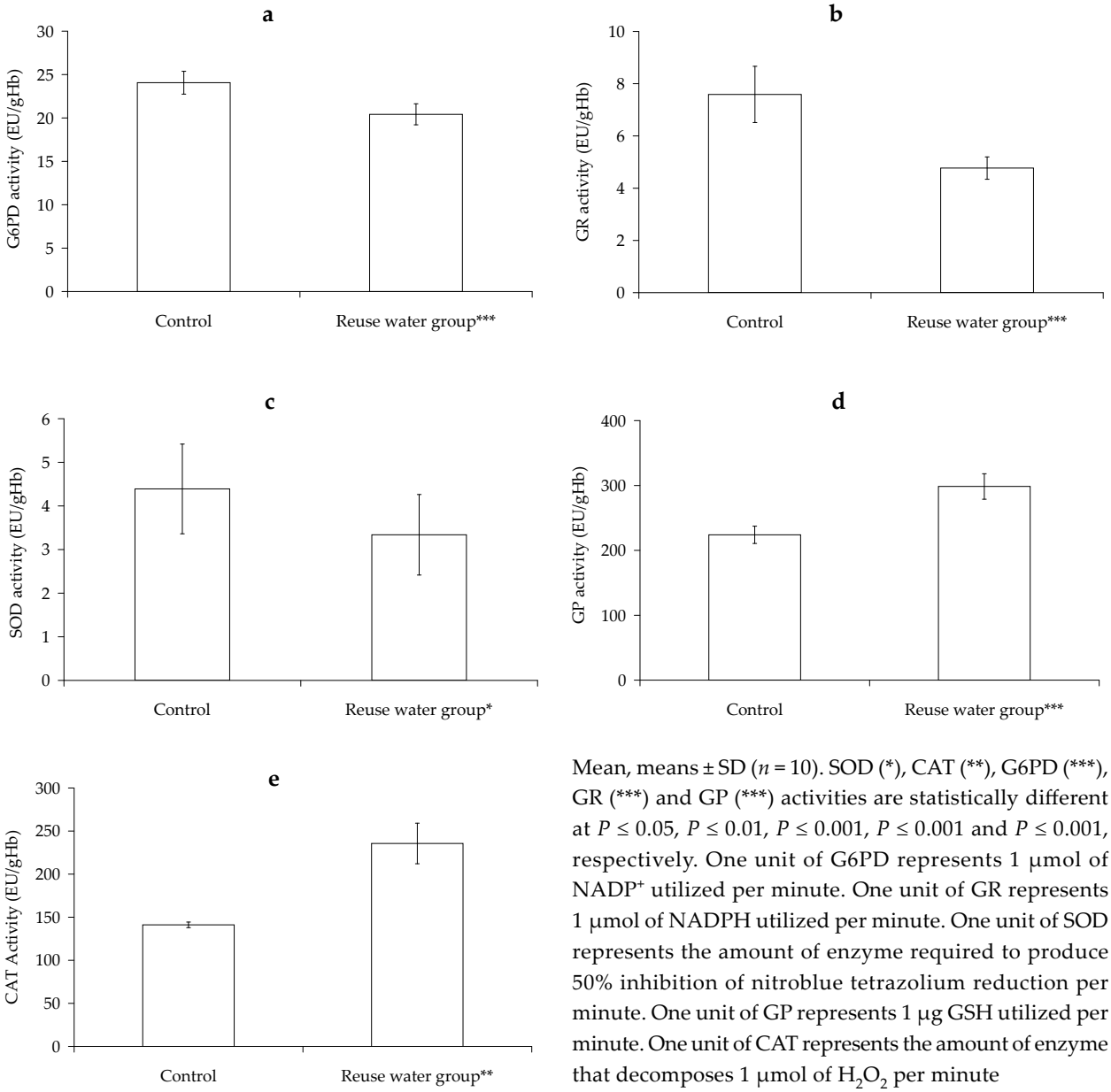
It is understood that the G6PD and GR activity were decreased ($P \leq 0.001$, 84.7% of control and $P < 0.001$, 63.2% of control, respectively) significantly in the reuse water group. A decrease in GR activity may lead to a decrease in reduced glutathione. The GSH/GSSG in normal cells is kept high, because of the reduction of GSSG back to GSH by GR enzyme. This depletion in GR activity which catalyses NADPH-dependent conversion of GSSG to GSH could be due to a decrease in NADPH levels, a secondary manifestation of cellular free radical stress (Chance and Boveris, 1980). The reduced availability of NADPH could be due to reduced synthesis in HMP resulted due to decreased activity G6PD as this enzyme plays a very important role to maintain high ratio of NADPH/$NADP^+$ in the cell and plays a crucial role in the regeneration of GSH from GSSG (Jain, 1998; Ozmen, 2004). A decrease was observed in the activity of G6PD in the treated groups, possibly attributable to blocked enzyme synthesis. NADPH is crucial in the protection of cells from oxidative stress caused by free radicals to a number of molecules in cells, including membrane lipids, proteins, and nucleic acids (Ames et al., 1993; McCord, 1993). If the generation of free radicals in cells impairs antioxidant defenses or exceeds the ability of the antioxidant defense system to eliminate them oxidative stress causes (Jenkins and Goldfarb, 1993; Ozmen and Kufrevioglu, 2004).

Table 1. Statistical values obtained from studies for flow-through and reuse water groups

Enzymes (EU/g HB)	Control (mean ± SD)	Reuse water group (means ± SD)
G6PD	24.1 ± 1.3	20.4 ± 1.2***
GR	7.59 ± 1.08	4.8 ± 0.43***
SOD	4.4 ± 1.03	3.34 ± 0.9*
GP	224 ± 13.3	298.5 ± 19.6***
CAT	141.2 ± 3.2	235.6 ± 23.6**

*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

SOD activity was significantly decreased in all the samples of reuse water group ($P < 0.05$, 76% of control). A fall in the activity of SOD may be due to inactivation by interaction with oxygen radicals. Hodgson and Fridovich (1975) and (Pigeolet et al. (1990) reported the decreased SOD by hydroxyl radical and H_2O_2. The depression in SOD activity may result in cellular injury by superoxide radical.



Mean, means ± SD (n = 10). SOD (*), CAT (**), G6PD (***), GR (***) and GP (***) activities are statistically different at $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, $P \leq 0.001$ and $P \leq 0.001$, respectively. One unit of G6PD represents 1 μmol of $NADP^+$ utilized per minute. One unit of GR represents 1 μmol of NADPH utilized per minute. One unit of SOD represents the amount of enzyme required to produce 50% inhibition of nitroblue tetrazolium reduction per minute. One unit of GP represents 1 μg GSH utilized per minute. One unit of CAT represents the amount of enzyme that decomposes 1 μmol of H_2O_2 per minute

Figure 2. Activities (means ± SD) of G6PD (a), GR (b), SOD (c), GP (d) and CAT (e) in erythrocytes of rainbow trout held in flow-through and reused water

GP and CAT activities increased ($P < 0.001$, 133% of control and $P < 0.01$, 178% of control, respectively) in the reuse water group. The increased activities of both CAT and GP in fish maintained in reuse water indicate the highly induced capacity to scavenge hydrogen peroxide produced in the red cells in response to oxidative stress. This situation may reflect the persistent oxidative stress. The increased GP and CAT activities may be a compensatory mechanism to get rid of excess peroxides (Schlorff et al., 1999). A similar trend was reported (Kolayli and Keha (1999) between fresh water and seawater-adapted rainbow trout. It is also reported increase in CAT activity of fish erythrocytes in California Bight where contamination is higher, and an increase in GP activity in human and fish (Dicentrarchus labrax) erythrocytes *in vitro* after treatment with copper and mercury ions has been also reported (Brown et al., 1987).

The results presented in this work clearly show that the fish resident in reuse water are under an oxidative stress condition, as shown in red cells

studied. We conclude that recirculation systems may be lead to oxidative stress. It has effects on the antioxidant defense system. This situation may be associated with an increased influx of free radicals and also fish become more sensitive to diseases, loss adaptation capabilities to different water conditions.

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Corresponding Author

Dr. Ismail Ozmen, Ataturk University, PTT Mudurlugu PK. 44, 25171 Erzurum, Turkey
Tel. +90 442 231 1680, fax +90 442 236 0948, e-mail: iozmen@atauni.edu.tr