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Melatonin administration increases antioxidant enzymes activities and reduces lipid peroxidation in the rainbow trout (*Oncorhynchus mykiss*, Walbaum) erythrocytes

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Abstract: Effects of melatonin on the levels of antioxidant enzymes including catalase (CAT), peroxidase (POD), glutathione reductase (GRd), and malondialdehyde (MDA), a lipid peroxidation marker, in erythrocytes of rainbow trout (*Oncorhynchus mykiss*) were investigated. The results showed that melatonin statistically activated the CAT, POD, and GRd activity ($P < 0.05$). Moreover, MDA levels were significantly decreased as a result of melatonin treatment ($P < 0.01$).

Key Words: Rainbow trout; melatonin; antioxidant enzyme; lipid peroxidation

Melatonin uygulaması gökkuşağı alabalık (*Oncorhynchus mykiss*, Walbaum) eritrositlerinde antioksidan enzim aktivitelerini artırırken lipit peroksidasyonunu azaltmaktadır

Özet: Çalışmada, melatoninin gökkuşağı alabalık eritrositlerindeki katalaz (CAT), peroksidaz (POD) ve glutatyon redüktaz (GRd) gibi antioksidan enzimler ile lipid peroksidasyonu göstergesi olan malondialdehit (MDA) üzerine etkileri araştırıldı. Sonuçta, melatonin uygulamasının CAT, POD ve GRd aktivitelerini istatistiksel olarak artırdığı ($P < 0,05$) gösterildi. Ayrıca melatonin uygulamasının bir sonucu olarak MDA seviyesinin önemli derecede azaldığı ($P < 0,01$) belirlenmiştir.

Anahtar Sözcükler: Gökkuşağı alabalığı; melatonin; antioksidan enzimler; lipit peroksidasyonu

Introduction

Melatonin is primarily produced by photoreceptor cells, which act as a photoneuroendocrine transducer and secrete melatonin into the blood, in the pineal gland (1). It is involved in many physiological processes, such as seasonal reproduction, activity rhythms, and sleep/wake cycles (2), in osmoregulation and/or stress adaptation in seawater (3). Moreover, it

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is considered as a powerful free radical scavenger and likely to be a general promoter of anti-oxidative mechanisms and a potential antioxidant in vitro and in vivo (4). Some of its interactions with other hormones such as cortisol and arginine vasotocin (hormones closely associated with electrolyte balance) have been reported in teleosts (5,6).

The antioxidant defense (AD) system of organisms provides a means of dealing with oxidative stress and includes several enzymes and vitamins. A primary role of the AD system is protecting cellular components from damage induced by reactive oxygen species (ROS) (7). The ROS include free radicals, such as superoxide anion radicals ($O_2^{\bullet-}$), hydroxyl radicals (OH·) and non-free radicals such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). Also, excessive generation of ROS induced by various stimuli and exceeding the antioxidant capacity of the organism leads to a variety of pathophysiological processes. Exposure of organisms to pro-oxidant attack can increase antioxidant defenses, e.g. by increasing synthesis of antioxidant enzymes (8). If antioxidant defenses are effective in detoxifying ROS, then no harmful consequence will result in the tissues. However, if the ROS attack is severe, antioxidant defense systems may be overwhelmed, resulting in inhibition of antioxidant enzymes and oxidative damage to lipids, proteins, 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 (9). Increased concentration of lipid peroxidation and DNA damage has been used as indicators of ROS-derived damage in biological systems (10).

Most cellular antioxidants can be regarded as part of the innate nonspecific immune system (11). Like other vertebrates, fish possess an AD system both enzymatic and non-enzymatic. The more relevant AD enzymes consist of glutathione reductase (GRd), superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), and non-enzymatic defenses include vitamin C, α-tocopherol, and vitamin A (12).

Although, the influence of melatonin administration on fish reproductive parameters has been extensively investigated (13,14), studies related to the effects of this application on the inhibition or activation of enzymes playing a key role in antioxidant processes are unfortunately scarce in fish. Therefore, the aim of the present study was to determine the effects of melatonin on the activity of endogenous antioxidant enzymes such as CAT, POD, and GRd, and the levels of MDA as a marker of peroxidized lipid concentrations from rainbow trout (*Oncorhynchus mykiss*) erythrocytes in vivo.

Materials and Methods

In vivo experiment

Fish treatments were conducted according to the Applied Research Ethics National Association (15). In the experiment, fish (mean weight: 200 ± 20 g) were divided into 2 groups as control and melatonin. Each included 20 fish. Fish were anaesthetized in water containing tricaine methanesulphonate (MS-222, 1/10,000), and 0.5 ml blood samples from the control group and the melatonin group were collected from the caudal sinus using heparinised syringes and placed into a heparinized vacutainer to determine initial activities of CAT, POD, and GSSG-Px as well as the levels of total MDA. Subsequently, melatonin was dissolved in a small amount of ethanol and then diluted with teleost saline (20 mg Na_2CO_3/100 ml of 0.6% NaCl) until the desired final concentrations were obtained. Physiological saline was injected to all fish in the control group intraperitoneally (i.p.). For the melatonin group, 10 mg/kg (fish body weight) melatonin was injected i.p. Blood samples were taken from the different fish at 1, 3, and 5 h after injection from the control and the melatonin group (n = 5). All blood samples were centrifuged at 2500 rpm for 15 min, then the plasma was removed by drip, the erythrocyte pellet was washed with 0.16 M KCl 3 times and the supernatant was discarded. One volume from the resulting erythrocyte pellet was hemolyzed in 5 volumes of ice-water. Ghost and intact cells were then removed by high-speed centrifugation (20000 rpm for 30 min, Heraeus Sepatech, Suprafuge). As a result, a hemolysate was prepared. Hemoglobin (Hb) concentration in hemolysate was determined by the cyanmethemoglobin method. All of the above-mentioned procedures were carried out at +4 °C.

Measurement of antioxidant enzyme activities

Catalase activity was assayed by monitoring the decrease in absorbance at 240 nm due to H_2O_2

consumption (16). The reaction mixture contained 0.1 ml hemolysate, 0.5 ml 10 mM H_2O_2 and 0.9 ml 30 mM potassium phosphate buffer (pH 7.3) and reduction in absorbance was recorded at 240 nm for 60 s. The enzyme activity is expressed as mmoles H_2O_2 decomposed by using the H_2O_2 extinction coefficient 36 $mM^{-1}.cm^{-1}$.

Peroxidase activity was measured by the method of Shannon et al. (17). For this purpose, 0.1 ml hemolysate were taken to 1.7 ml 0.05 M potassium phosphate buffer (pH 7.3). Then 0.5 ml ABTS were added. The reaction was started by the addition of 0.2 ml 0.2 M H_2O_2. Change in absorbance at 470 nm was recorded for 2 min at intervals of 15 s. The enzyme activity is expressed as enzyme unit $min^{-1}gHb^{-1}$.

GRd activity was estimated by the method of Goldberg and Spooner (18). To 0.1 ml hemolysate 2.5 ml 120 mM potassium phosphate buffer (pH 7.3), 0.1 ml 0.015 mM EDTA, and 0.1 ml 0.065 mM oxidized glutathione were added. After 5 min, 0.05 ml 9.6 mM NADH were added and mixed. The absorbance was recorded at 340 nm at 15 s intervals. The enzyme activity is expressed as nmole NADH oxidized $min^{-1}.gHb^{-1}$ using the molar extinction coefficient 6.23 $mM^{-1}cm^{-1}$.

Measurement of MDA level

MDA levels of rainbow trout erythrocytes were estimated according to the modified method of Hunter et al. (19). To 0.5 ml hemolysate, 0.5 ml Tris/HCl buffer (50 mM, pH 7.4) was added followed by further mixing and incubation at room temperature for 10 min; 1.0 ml of 0.75% thiobarbituric acid in 2 M Na_2SO_4 was added and then the mixture was heated to 100 °C for 45 min. After cooling, 1.0 ml of 70% TCA was added, the mixture was vortexed, and than centrifuged at 1000 × g for 10 min. The absorbance of supernatant was determined at 530 nm. Total thiobarbituric acid-reactive materials are expressed as MDA, using a molar extinction coefficient for MDA of $1.56 \times 10^5\ cm^{-1}M^{-1}$.

Statistical analyses

The obtained data were analyzed by Student's t-test or one way analysis of variance (ANOVA), followed by Tukey multiple range test to determine significant differences among means at the α: 0.05 level.

Results

In the present study, it was observed that there were no differences ($P > 0.05$) between the enzyme activities determined in the control group at 0 (initial), 1, 3, and 5 h for each enzyme. However, as can be seen in the Table, the differences between the mean values of enzyme activities determined at different times were statistically significant ($P < 0.05$, using the ANOVA). Melatonin increased the CAT activities in rainbow trout at 1 h as compared to the control values. These differences were found to be significant ($P < 0.001$, using Student's t-test). However, CAT activity returned to the control level at 5 h after treatment ($P > 0.05$). Like CAT activity, POD and GRd activities were increased after melatonin injection. Maximal increases of POD and GRd activities by the fish dose of melatonin occurred within 1 h after melatonin administration ($P < 0.01$, using Student's t-test). POD and GRd activities returned to the control level at 3 h and 5 h, respectively after treatment ($P > 0.05$) (Figure 1).

It was observed that there were no differences ($P > 0.05$) between the MDA levels determined in the control group at 0 (initial), 1, 3, and 5 h. The decreased concentrations of MDA in the rainbow trout plasma were found after treatment with a 10 $mg.kg^{-1}$ dose of melatonin. Melatonin especially reduces lipid peroxidation after 5 h (Figure 2). This difference was found to be statistically significant ($P < 0.01$, using Student's t-test).

Table. The effect of melatonin on the activities of antioxidant enzymes from rainbow trout (*Oncorhynchus mykiss*) erythrocytes (CAT: catalase, POD: peroxidase GRd: glutathione reductase, EU: enzyme unit, Hb: haemoglobin).

Time (h)	**CAT** (EU/gHb)	**POD** (EU/gHb)	**GRd** (EU/gHb)
0	462.8 ± 30.3^a	1339.2 ± 243.3^a	11.9 ± 2.3^a
1	564.4 ± 28.5^b	1809.3 ± 164.6^b	26.9 ± 4.4^b
3	616.2 ± 48.1^b	1532.5 ± 269.4^a	16.4 ± 2.4^a
5	421.3 ± 69.5^a	1140.0 ± 376.3^a	12.9 ± 3.2^a

Values are means ± SD of 5 fish

Means in a column with different superscripts differ significantly ($P < 0.05$)

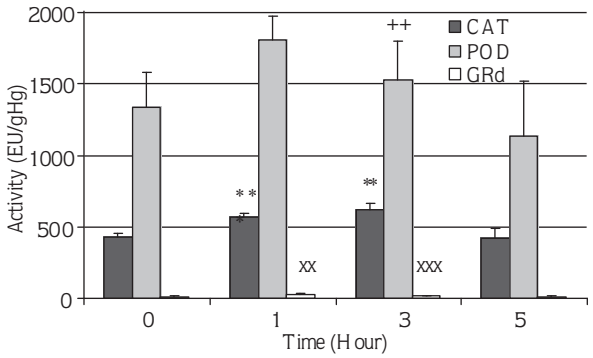

Figure 1. Changes of antioxidant enzymes activities (EU/gHb) in rainbow trout (*Oncorhynchus mykiss*) erythrocytes after the administration pharmacological doses of melatonin (10 mg kg^{-1}) (differences relative to time 0 h: *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001 versus control using Student's t-test).

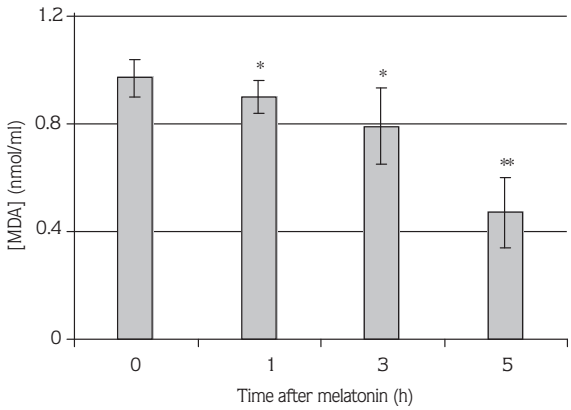

Figure 2. Levels of malondialdehyde (MDA) in rainbow trout (*Oncorhynchus mykiss*) plasma after the administration pharmacological doses of melatonin (10 mg kg^{-1}) (differences relative to time 0 h: *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001 versus control using Student's t-test).

Discussion

Melatonin and its metabolites are known to prevent oxidative damage to the cell membrane, cytosolic organelles, and nuclear and mitochondrial DNA by electron donation (20,21). These effects can occur by at least 2 mechanisms. In one case, melatonin itself exerts direct antioxidant effects via free-radical scavenging and/or by inhibiting the generation of these toxic reactants. Additionally, melatonin alters the activities of antioxidant enzymes in mammals (22).

A variety of studies have demonstrated an improvement not only in oxidative stress parameters measured in plasma, serum, liver, kidney, and brain, but also in scavenger enzyme activities in other experimental models after melatonin is administered (23). In recent studies, the effect of melatonin on carbonic anhydrase enzyme activity in rainbow trout erythrocytes was determined as in vitro and in vivo. It was observed that melatonin did not inhibit the rainbow trout erythrocyte carbonic anhydrase enzyme activity in vitro. On the contrary, the results did show that rainbow trout erythrocyte carbonic anhydrase was inhibited significantly by melatonin after its injection in vivo ($P < 0.05$) (24). Furthermore, it has been documented that melatonin has an effect on glucose-6-phospate dehydrogenase from rainbow trout erythrocytes. In vivo studies showed that, though initial glucose-6-phospate dehydrogenase activity was 8.33 EU/gHb, these values fell after the injection of a pharmacological dose of melatonin (25).

Oxidation of membrane lipids can lead to the loss of cellular or organelle membrane integrity, which can eventually result in membrane rigidity, pathological injury, and cell death (26). Salmonid tissues are characterized by high concentrations of polyunsaturated fatty acids compared with most mammalian tissues, and fish may, therefore, be particularly susceptible to lipid peroxidative cellular damage (27). In previous studies, melatonin levels were found to correlate with the total antioxidant capacity of rat (28) and human serum (29) and in all studies using mammals, melatonin characteristically depressed the products of lipid peroxidation.

In conclusion, our study showed that melatonin reduced levels of lipid peroxides and increased the activity of antioxidant enzymes such as CAT, POD, and GRd in rainbow trout. These finding are consistent with other published reports using other species (22,30).

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