

## Effects of dietary melatonin supplementation on some blood oxidative status biomarkers and biochemical parameters in common carp (*Cyprinus carpio*)

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### Abstract

This study was conducted to evaluate the effect of dietary melatonin supplementation (10 mg/kg body wt. for 4 weeks) on some erythrocytic antioxidant enzymes and lipid peroxidation as well as some plasma biochemical parameters in common carp. The results showed that the activities of glutathione peroxidase and superoxide dismutase were increased significantly ( $p < 0.05$ ) after melatonin supplementation, whereas alterations in catalase activity were not significant. Malondialdehyde concentrations in erythrocyte hemolysate were decreased significantly as a result of melatonin treatment. Plasma biochemical analysis revealed a significant decrease of glucose, cholesterol, albumin and triglyceride, in addition to a significant increase in total protein, AST and ALP values when compared to the control group. However, alterations in creatinine, urea and LDH levels were not significant following melatonin supplementation compared to the control values. These results offer that pharmacological amounts of melatonin effectively improve erythrocytic oxidative status biomarkers and also alter some plasma biochemical parameters in the common carp that some of them may be undesirable. However, more investigations are required to elucidate the pharmacokinetic effects of melatonin and also possible undesirable effects of high doses of this compound.

**Keywords:** Melatonin, oxidative status biomarkers, biochemical parameters, common carp

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## Introduction

Formation of free radical species by the partial reduction of oxygen is an inevitable consequence of aerobic metabolism and is potentially injurious to cells. Under physiological conditions there is continuous production of reactive oxygen species (ROS), particularly in mitochondria, microsomes, nuclear membranes and phagocytes (Halliwell and Gutteridge, 1996). The extant antioxidant defense systems inhibit oxidative damage to cells. Oxidative stress resulting from increased production of free radicals and reactive oxygen species (ROS), and/or a decrease in antioxidant defense, leads in impairment of DNA, enzymes and membranes disorders and induces changes in the activity of the immune system as well as in the structure of basic biopolymers which, in turn, may be related to various disorders (Abd Ellah, 2010). The deleterious effects resulting from the cellular oxidative state may be alleviated by enzymatic and nonenzymatic antioxidant machinery that vary at various cellular and subcellular levels in fish. Fish use a diverse array of enzymes like superoxide dismutase, glutathione peroxidase and catalase, as well as low molecular weight antioxidants like glutathione and ascorbic acid to scavenge different types of reactive oxygen species (Almeida *et al.*, 2002; Roberts and Oris, 2004; Feng *et al.*, 2013).

Trends on applying various antioxidant compounds for prevention or protection of oxidative damages caused by free radicals have gained immense interest in recent years. In both mammals and fish, insufficient dietary antioxidants have been followed by a decrease in antioxidative defense and increased susceptibility to oxidative stress (Sies *et al.*, 2005; Welker and Congleton, 2009). The antioxidative defense system capacity of cultured fish has been found insufficient (Nakano *et al.*, 1999). With regard to increasing water pollution and its stimulating effects on oxidative damage to fish tissues and also in view of the high content of polyunsaturated fatty acids in fish tissues, improving antioxidative status of fish seems to be necessary and may be associated with beneficial effects on health status of fish (Mohebbi *et al.*, 2011).

Melatonin (N-acetyl-5-methoxytryptamine) has been known for a long time as the major pineal secretory product but later it has emerged as a compound that can also be synthesized in other organs and tissues such as eyes, gastrointestinal tract, bones, skin, lymphocytes, platelets, and thymus (Reiter *et al.*, 2007), and also has now been shown to exist in the plant kingdom, including edible plants, bacteria, unicellular eukaryotes, and invertebrates (Reiter and Tan, 2002). Many studies have shown that melatonin and its metabolites to be highly effective free radical scavengers and ubiquitously acting antioxidants, which play an essential role in reducing oxidative stress under a variety of experimental settings (Tan *et al.*, 2000; Martinez *et al.*, 2002; Reiter and Tan, 2002; Tan *et al.*, 2007). It has been reported that melatonin is more powerful than glutathione in neutralizing free radicals and also it can protect cell membranes from oxidative damage more effectively than vitamin E (Reiter *et al.*, 1997). Since the concentration of melatonin in the blood correlates positively with the total antioxidant status of this fluid (Benot *et al.*, 1999), the implication is that consuming foodstuffs containing melatonin would increase the antioxidative capacity of the organism (Reiter and Tan, 2002). Numerous studies support beneficial effects of melatonin on metabolic and oxidative status profile in various tissues of mammals (Cruz *et al.*, 2001; Reiter and Tan, 2002; Tunes *et al.*, 2002; Anwar and Meki, 2003; Subramanian *et al.*, 2007), but to our knowledge studies related to the melatonin effects on oxidative status biomarkers and plasma biochemistry in fish species is scarce.

The freshwater fish, the common carp (*Cyprinus carpio*), has great commercial importance because it is the most common fish that is widely consumed worldwide. The present study was conducted to assess the effects of dietary melatonin supplementation on some blood oxidative status biomarkers and biochemical variables in common carp.

## Materials and methods

### *Chemical Materials*

Melatonin was purchased from Nature Made

(USA). Commercial enzyme kits for superoxide dismutase (Ransod, RANDOX/SD-125) and glutathione peroxidase (Ransel, RANDOX/RS-505) were obtained from Randox Laboratories (UK). Commercially available kits (Pars Azmoon, Iran) were used for measuring plasma biochemical parameters. MS-222 (Ethyl 3-aminobenzoate methanesulfonate, Tricaine) and 2-thiobarbituric acid (TBA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The rest of the utilized chemicals were of analytical grade and were supplied by Sigma (St. Lewis, MO, USA) or Merck (Darmstadt, Germany).

### *Experimental Design*

Common carp (*C. carpio*; total n=60), weighing 60-80 g, were obtained from a local commercial farm. They were held in six glass aquaria, each containing 250 l fresh water. Fish were acclimatized for 7 days before the commencement of the experiment and were fed daily with commercial fish food at 3% total body weight at a fixed time.

Physicochemical conditions of the water during the experimental period were as following dissolved oxygen, 5.5–6 ppm; temperature, 25±1°C; pH, 7±0.5. Photoperiod was a 12:12 light–dark cycle. The water in the aquariums was renewed every 48 h. Fish were divided randomly into two groups of 30 each (3 aquaria considered as control and 3 aquaria considered as treatment). Group 1 fish were fed with basic diet; served as control. Group 2 fish were fed the basic diet supplemented with melatonin (10 mg/kg body weight, daily) for 4 weeks.

### *Blood sampling and processing*

At the end of the experimental period, twenty fish were collected randomly from each aquarium and anesthetized in diluted MS-222. Blood samples were taken in heparinized tubes. After plasma separation by centrifugation at 750 g for 20 min, erythrocyte pellet was washed three times with normal saline solution. The washed centrifuged erythrocytes were haemolysed by the addition of an equal volume of ice-cold redistilled water and prepared haemolysate and plasma

aliquots were stored at -70°C until analysis.

### *Biochemical assays and analysis*

Glutathione peroxidase (GPx) activity was measured using RANDOX-Ransel enzyme kit, and the results were expressed as units per gram hemoglobin. Hemoglobin concentration was measured by cyanmethemoglobin method.

Superoxide dismutase (SOD) activity was determined by a modified method of iodophenyl nitrophenol phenyltetrazolium chloride using the RANDOX-Ransod enzyme kit, and the results were expressed as units per gram hemoglobin.

Catalase (CAT) activity was measured in the RBC haemolysate by the method described by Claiborne (1986) and expressed as units per gram hemoglobin. The decomposition of H<sub>2</sub>O<sub>2</sub> can be directly followed by the decrease of absorbance at 240 nm. The difference in absorbance at 240 nm per time unit allows determining the CAT activity.

Determination of malondialdehyde (MDA) concentration was based on spectrophotometry of the pink colored product of thiobarbituric acid reactive substances, as described by Latha and Pari (2003). The concentration of MDA was calculated using a molar extinction coefficient value of 156,000 M<sup>-1</sup> cm<sup>-1</sup>. The results were expressed as nanomoles of MDA per gram hemoglobin.

Plasma biochemical analysis including glucose, triglyceride, cholesterol, total protein, albumin, urea, creatinine, alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) were measured using commercial colorimetric kits (Pars Azmoon, Iran).

### *Statistical analysis*

All experimental values have been represented as mean ± standard error of mean (SEM). The obtained data were analyzed using Student's t-test. The level of significance was set at P < 0.05. All calculations were performed using SPSS/PC software.

## **Results**

The effects of dietary melatonin

supplementation on erythrocyte oxidative status biomarkers and plasma biochemical parameters in experimental groups are presented in Tables 1 and 2, respectively. As shown in Table 1, the activities of GPx and SOD were increased significantly following melatonin supplementation by approximately 31.4% for GPx and 13.2% for SOD in comparison with control group values. However, alterations in catalase activity were not significant. Supplementation with melatonin was effective to decrease the MDA concentration in erythrocyte hemolysate by approximately 36% in comparison to control values.

As shown in Table 2, the concentrations of glucose, cholesterol, albumin and triglyceride were decreased significantly following melatonin supplementation approximately 18.6%, 59.1%, 24.8 % and 27.7 %, respectively, in comparison with control group values. Additionally, total protein, AST and ALP values were increased significantly about 19.6%, 49.7% and 71.6%, respectively, in group 2 as compared to control group values (Table 2). However, alterations in creatinine, urea and LDH levels were not significant following melatonin supplementation compared to control values.

**Table 1. Mean  $\pm$ SEM of measured oxidative status biomarkers in experimental groups (n= 20 in each group).**

Parameter	Control	Melatonin
Glutathione peroxidase (U/g Hb)	744.96 $\pm$ 38.37 <sup>a</sup>	978.94 $\pm$ 95.27 <sup>b</sup>
Superoxide dismutase (U/g Hb)	1573.67 $\pm$ 68.53 <sup>a</sup>	1780.98 $\pm$ 47.65 <sup>b</sup>
Catalase (U/g Hb)	273.41 $\pm$ 30.95	238.84 $\pm$ 14.63
Malondialdehyde (nmol/g Hb)	129.29 $\pm$ 10.37 <sup>a</sup>	82.77 $\pm$ 8.58 <sup>b</sup>

<sup>a, b</sup> Mean  $\pm$  SEM in each row with no common superscript differ significantly ( $P < 0.05$ ).

**Table 2. Mean  $\pm$  SEM of measured plasma biochemical parameters (Mean  $\pm$ SEM) in experimental groups (n= 20 in each group).**

Parameter	Control	Melatonin
Glucose (mg/dl)	41.32 $\pm$ 1.44 <sup>a</sup>	33.65 $\pm$ 2.62 <sup>b</sup>
Cholesterol (mg/dl)	164.54 $\pm$ 7.12 <sup>a</sup>	67.28 $\pm$ 8.12 <sup>b</sup>
Triglyceride (mg/dL)	101.00 $\pm$ 10.87 <sup>a</sup>	73 $\pm$ 6.20 <sup>b</sup>
Total protein (g/dl)	3.31 $\pm$ 0.06 <sup>a</sup>	3.96 $\pm$ 0.05 <sup>b</sup>
Albumin (g/dl)	1.41 $\pm$ .15 <sup>a</sup>	1.06 $\pm$ 0.18 <sup>b</sup>
Creatinine (mg/dl)	0.44 $\pm$ .02	0.40 $\pm$ 0.02
Urea (mg/dl)	4.15 $\pm$ 0.27	4.65 $\pm$ 0.44
Aspartate aminotransferase (IU/L)	48.20 $\pm$ 3.64 <sup>a</sup>	72.15 $\pm$ 9.88 <sup>b</sup>
Lactate dehydrogenase (IU/L)	115.20 $\pm$ 8.22	117.55 $\pm$ 18.30
Alkaline phosphatase (IU/L)	37.20 $\pm$ 5.50 <sup>a</sup>	63.85 $\pm$ 4.80 <sup>b</sup>

<sup>a, b</sup> Mean  $\pm$  SEM in each row with no common superscript differ significantly ( $P < 0.05$ ).

## Discussion

Oxidative stress is an active field of research in medicine and has been implicated in numerous pathological conditions in both humans and animals. Numerous studies have been carried out to investigate the effects of various antioxidants on the oxido-reductive status of different tissues. In this regard, assay of circulatory biomarkers of oxidative stress has emerged as a reliable method for screening putative chemopreventive agents (Balasenthil *et al.*, 2000). In the present study, dietary melatonin supplementation was found to

have beneficial effects on erythrocytic oxidative status biomarkers and also altered some plasma metabolic parameters in the common carp.

Fish erythrocytes have been proposed as a useful model to investigate oxidative stress, since their membranes are rich in long chain polyunsaturated fatty acids, which are oxidized under oxidative stress conditions induced by metals or other compounds (Roche and Boge, 1993; Gabryelak *et al.*, 2000; Nagasaka *et al.*, 2004). Moreover, repeatedly exposure to high concentration of oxygen or presence of iron

renders erythrocytes highly susceptible to peroxidative damage (Clemens and Waller, 1987). Among the known biological molecules, lipids are one of the most susceptible substrates to free radicals damage and biomarkers of lipid peroxidation are considered the best indicators of oxidative stress (Georgieva, 2005). Lipid peroxidation is a non-enzymatic chain reaction based on oxidation of mainly unsaturated fatty acids and is associated with the presence of reactive oxygen species. The extent of lipid peroxidation is most frequently measured by estimating MDA levels (Lata *et al.*, 2004). Fish tissues are characterized by high concentrations of polyunsaturated fatty acids and may therefore be particularly susceptible to lipid peroxidation (Winston and DiGiulio, 1991). The decreased levels of erythrocytic MDA levels found in the present study after taking dietary melatonin supplementation in the common carp suggest that melatonin may provide an effective protection against lipid peroxidation. In agreement with this finding, it has been reported that melatonin significantly decreased the products of lipid peroxidation in different tissues of rats (Subramanian *et al.*, 2007). It has also been reported that intraperitoneal injection of 10 mg/kg melatonin results in a significant decrease in plasma MDA concentrations in the rainbow trout after 1, 3 and 5 hours of injection in comparison to control group (Gulcin *et al.*, 2009). Similarly, it has been reported that dietary vitamin E supplementation caused a significant decrease in the levels of tissue lipid peroxides in three fish species (Tocher *et al.*, 2002). Baydas *et al.* (2002) compared vitamin E and melatonin effects on brain, liver and kidney MDA levels in streptozotocin-induced diabetic rats and found that MDA levels are more efficiently decreased with administration of melatonin compared to vitamin E, suggesting that melatonin seems to be a more potent antioxidant, especially in the brain and kidney. Moreover, Vural *et al.* (2001) and Anwar and Meki (2003) showed a significant return of erythrocytic MDA levels in the melatonin-treated diabetic group in comparison to the untreated diabetic group to approximate levels of the control group. However, in another study

the decrease in the testicular tissue levels of MDA was not significant in the melatonin-treated diabetic group compared to the untreated diabetic group (Armagan *et al.*, 2006). Significant decrease in thiobarbituric acid reactive substances has been reported in rat hippocampus after taking melatonin administration (Gonenc *et al.*, 2005).

Assaying antioxidant enzymes is among the most widely used methods for determination of oxidative stress. The detoxification of reactive oxygen species involves the cooperative action of the intracellular antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase. The seleno-enzyme glutathione peroxidase contributes to the oxidative defense of animal tissues by catalyzing the reduction of hydrogen and lipid peroxides. Catalase has an equal importance to GPx in the defense of human erythrocytes against H<sub>2</sub>O<sub>2</sub> generating reactions (Kaneko *et al.*, 1997). Superoxide dismutase is also important in the antioxidative defense mechanism and protects against lipid peroxidation (Miller *et al.*, 1993). In the present study the activities of superoxide dismutase and glutathione peroxidase in the erythrocytes of melatonin-supplemented carp were found to be significantly increased. These findings are in agreement with those represented by Subramanian *et al.* (2007) which melatonin at both doses of 0.5 and 1.0 mg/kg (intraperitoneal injection for 45 days) result in significant increase in the activities of SOD and GPx in the brain and liver of male Wistar rats. In addition, it has been reported that melatonin increased the activity of hepatic superoxide dismutase, glutathione peroxidase and glutathione reductase in the experimental biliary obstruction in rats (Montilla *et al.*, 2001). Similarly, Anwar and Meki (2003) have also reported that SOD activity increases significantly after taking melatonin treatment in liver and kidney homogenates (but not in erythrocyte lysate) of diabetic rats. It has been also found that exogenously administered melatonin cause a 2-fold rise in glutathione peroxidase activity during 30 minutes in the brain of the rat (Barlow *et al.*, 1995). Similarly, melatonin administration caused significant increase in GPx activity, but not SOD, in the rat

hippocampus (Gonenc *et al.*, 2005).

According to our results, catalase activity was not significantly affected following melatonin supplementation in common carp. In line with this finding, Anwar and Meki (2003) showed that melatonin administration had no significant effects on the catalase activity in erythrocyte lysate, liver and kidney of diabetic rats. In contrast, Subramanian *et al.* (2007) reported significant increase of catalase activity after melatonin treatment in examined tissues of rats. Additionally, it has been reported that melatonin administration increases catalase activity in the rainbow trout erythrocytes (Gulcin *et al.*, 2009). Similarly, vitamin E supplementation caused notable increase in erythrocyte catalase activity in humans (Brown *et al.*, 1996).

The possible mechanism underlying the antioxidant effects of melatonin, as noticed in the present study, could primarily be explained by at least two mechanisms. Melatonin possesses redox properties and considered as a highly effective free radical scavenger because of the presence of an electron rich system which allows this molecule to act as an electron donor (Reiter *et al.*, 1997; Allegra *et al.*, 2003). Melatonin may decrease oxidative stress also by stimulating some antioxidant enzymes (Martinez *et al.*, 2002; Reiter and Tan, 2002; Tan *et al.*, 2007). Another criterion, which gives an extra advantage to melatonin when considering its antioxidant potential, is its small size and lipophilicity, thus, it readily passes across biological membranes and reaches into every cell and also into all subcellular compartments of the cell including the mitochondria (Tan *et al.*, 2000; Reiter and Tan, 2002). Melatonin's presence in mitochondria may be of special importance since these organelles are a major source of free radicals and oxidative damage (Reiter and Tan, 2002).

According to our results melatonin administration caused significant decrease in glucose, cholesterol, and triglyceride concentrations. These findings are in agreement with Nishida *et al.* (2002) who reported that subcutaneous implantation of a melatonin releasing pellet resulted in improved lipid metabolism in diabetic rats. Moreover, treatment

of diabetic rats with melatonin decreased blood glucose, triglyceride, total lipids and cholesterol levels (Anwar and Meki, 2003). Findings of Derlacz *et al.* (2005) showed melatonin-induced modulation of glucose metabolism in primary cultures of rabbit kidney-cortex tubules. In rats, melatonin injection reduced serum cholesterol and prevented fatty degeneration of the liver in hypercholesterolemic animals (Aoyama *et al.*, 1988). Long term melatonin treatment may decrease cholesterol synthesis (Muller-Wieland *et al.*, 1994) and augment endogenous cholesterol clearance mechanisms (Chan and Tang, 1995). Moreover, Maitra *et al.* (2000) reported that melatonin may decrease blood glucose levels through its role on catecholaminergic responses.

In the present study, plasma AST and ALP activities were increased significantly following melatonin supplementation. This may suggest that the melatonin dose applied at the present study may have cytotoxic effects on liver cells of the common carp. In addition, reductions in plasma concentrations of some energy-related biomolecules may be attributed to disorders in metabolic functions of the liver following melatonin treatment. However, to get a better understanding of the undesirable effects of pharmacological doses of melatonin on circulating biochemical parameters, further studies are needed to verify the relationship between biochemical changes and possible histopathological lesions.

In summary, our study showed that dietary supplementation of melatonin appear to be able to protect the oxidoreductive status of erythrocytes in carp by increasing the activities of some antioxidant enzymes as well as decreasing lipid peroxidation. Moreover, melatonin might be considered as a factor affecting some circulating metabolic variables that some of these alterations may be undesirable. Nevertheless, more investigations are required to elucidate the pharmacokinetic effects of melatonin and also precise molecular basis of the beneficial effects of melatonin in fish species.

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## اثرات افزودن ملاتونین به جیره غذایی بر برخی بیومارکرهای وضعیت اکسیداتیو و فراسنجه های بیوشیمیایی خون در کپور معمولی (*Cyprinus carpio*)

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### چکیده

این مطالعه به منظور بررسی تاثیر افزودن ملاتونین به جیره (۱۰ میلی گرم بر کیلوگرم جیره به مدت ۴ هفته) بر برخی آنزیم های آنتی اکسیدانت و پراکسیداسیون لیپیدی در گلبول های قرمز و همچنین برخی فراسنجه های بیوشیمیایی پلاسما انجام گرفت. نتایج این تحقیق نشان داد که فعالیت گلوکاتایون پراکسیداز و سوپر اکسید دیسموتاز به دنبال افزودن ملاتونین افزایش معنی دار ( $P < 0.05$ ) داشتند در حالیکه تغییرات فعالیت کاتالاز معنی دار نبود. غلظت مالون دی آلدئید در همولیزات گلبول های قرمز به دنبال تجویز ملاتونین کاهش معنی دار نشان داد. یافته های بیوشیمیایی پلاسما نشان دهنده کاهش معنی دار گلوکز، کلسترول، آلبومین و تری گلیسرید و نیز افزایش معنی دار پروتئین تام، AST و ALP در مقایسه با گروه کنترل بود. البته تغییرات مقادیر کراتینین، اوره و LDH به دنبال افزودن ملاتونین در مقایسه با گروه کنترل معنی دار نبود. این یافته ها پیشنهاد می کند که مقادیر فارماکولوژیک ملاتونین به طور موثری بیومارکرهای وضعیت اکسیداتیو گلبول های قرمز را در ماهی کپور معمولی بهبود می دهد و همچنین تغییراتی در پارامتر های بیوشیمیایی پلاسما ایجاد می کند که برخی از آنها ممکن است نامطلوب باشند. به هر حال، تحقیقات بیشتر به منظور توضیح اثرات فارماکوکینتیک ملاتونین و نیز اثرات نامطلوب دوز های بالای این ترکیب مورد نیاز است.

**واژگان کلیدی:** ملاتونین، بیومارکرهای وضعیت اکسیداتیو، فراسنجه های بیوشیمیایی، کپور معمولی