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Effect of Melatonin on the Antioxidant Enzyme Status in the Liver of Swiss Albino Laboratory Mice in Single Circadian Rhythm

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Abstract

Melatonin has been shown to be an effective antioxidant in a number of experimental models both in vitro and in vivo. However, limited information is available on monitoring the effect of melatonin on the antioxidant enzyme status in the liver in single circadian rhythm. To monitor the effect of melatonin on antioxidant enzyme activity, we used Swiss albino inbred male mice as a model system. The crude enzyme fractions were prepared from freshly isolated livers at 0, 2, and 24 hours as light cycle and 4, 8, and 12 hours as dark cycle specimens, post injection of melatonin. The injection of melatonin resulted in a statistically significant decrease in activity of SOD compared to control group ($p<0.01$). However, harvesting time did not affect SOD levels significantly. The activity of CAT was significantly decreased by melatonin and this decrease was clearly related to the time of harvest ($p<0.01$). Inhibition on SOD activity started at the beginning of the dark cycle of the circadian rhythm, while CAT activity started to decrease at that point. The inhibition of CAT reached its maximum at 8 hour post injection. After 12 hour post injection, the beginning of the light cycle period; CAT activity turned back to the control level. We concluded that based on control data, internal melatonin release increases SOD and CAT activity. The external melatonin treatment directly inhibits mostly •OH radicals and H_2O_2 as well as singlet oxygen ($^1O_2$).

Key words: Melatonin, Liver, Antioxidant enzymes, Superoxide dismutase, Catalase

INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamin) is a secretory product of the pineal gland and is released in a cyclic manner. Melatonin is synthesized and secreted by the pineal gland during the dark period of the light-dark cycle and suppressed by light. One of the main roles of melatonin is its function in circadian rhythm regulation [1]. It is a ubiquitously acting, direct free radical scavenger and also an indirect antioxidant [2-4]. Melatonin, as a highly electro- reactive molecule, detoxifies electron-deficient reactive oxygen *species* by electron donation [5-7]. While melatonin is highly efficient in detoxifying the devastatingly toxic hydroxyl radical (•OH) and its precursor H_2O_2, evidence also suggests that it interacts with singlet oxygen ($^1O_2$), peroxynitrite anion ($ONOO^-$) and nitric oxide (NO•) [3, 4, 8, 9]. Besides these direct scavenging actions, melatonin augments the activities of several antioxidative enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GRx), thereby helps lower the oxidative burden of the cells [2, 10-12]. Therefore, melatonin has an increasing interest recently. The cells make a number of antioxidant enzymes to scavenge the dangerous side-effects of oxygen. Here, we focused on two important players of them that are superoxide dismutase, which converts superoxide radicals into hydrogen peroxide, and catalase, which converts hydrogen peroxide into water and gaseous oxygen.

Melatonin has also been reported to stimulate the activities of enzymes and increase gene expression that improves the total anti-oxidative defense capacity of the organism, i.e., SOD [13]. Moreover, recent studies indicate that melatonin is effective on inhibiting oxidative liver damage [14-17]. Melatonin also could dose-dependently reduce liver lipid peroxide content in CCl_4 treated rats [18]. Melatonin inhibits, oxidative stress caused by paraquat in rat liver. Paraquat is the commonly used herbicide that causes fatal intoxication in both humans and animals [19]. Based on these literature records, melatonin has been able to prevent liver, heart, lung, and brain damage induced by oxidative stress [2, 20-21].

Superoxide ($•O_2$) is one of the most harmful agents in the cell. SOD scavenges this pro-oxidative agent into oxygen and hydrogen peroxide. SOD is the key antioxidant enzyme that has an important physiological role in development. It has already been shown that lack of these enzymes in genetically modified mice causes severe pathologies. SOD is present in three forms, intracellular form mostly located in the cytoplasm and mitochondrial inner membrane SOD1 (CuZn SOD), which represents 85–90% of the total SOD, mitochondrial form located in mitochondrial matrix SOD2 (Mn SOD), a free radical scavenging enzyme that is the first line of defense against superoxide produced as a by-product of oxidative phosphorylation and an extra cellular form SOD3 (CuZn SOD) [22]. Mice lacking SOD1 (CuZn SOD) develop a wide range of pathologies,including

hepatocellular carcinoma [23], an acceleration of age-related muscle mass loss [24], an earlier incidence of cataracts and a reduced lifespan. In humans, mutations in SOD1 have been linked to familial amyotrophic lateral sclerosis. However, patients with Down's syndrome have higher expression of SOD1 [25]. SOD2 knock out mice die shortly after birth, with intense oxidative stress and dilated cardiomyopathy that can affect the lungs, liver, and other body systems [26]. Mice lacking SOD3 do not show any obvious defects and exhibit a normal lifespan [27].

Hydrogen peroxide is a devastating intermediate product of plenty of metabolic processes. It must be converted into less dangerous substances as soon as possible to keep cells healthy. The enzyme catalase (CAT) is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide into less reactive gaseous oxygen and liquid water molecules [28]. Catalases are antioxidant metalloenzymes, ubiquitous among aerobic organisms, which promote the conversion of hydrogen peroxide to water and molecular oxygen. Although, CAT plays a major role in cellular antioxidant defense by decomposing hydrogen peroxide, thereby preventing the generation of hydroxyl radical by the Fenton reaction, the underlying mechanisms of biological significance of catalase have not yet been completely understood. The results of the experiments with CAT knockout m ice indicate that, they are not vulnerable to oxidative damage of H_2O_2. The knockout mice are phenotypically normal, so it seems that catalase is dispensable for mice [29]. As mentioned above, a number of metabolic processes involving free radicals production are related to pathogenesis.

In the current study, we aimed to assess the effect of external melatonin treatment on the antioxidant enzymes, SOD and CAT, in the liver of inbred male Swiss albino laboratory mice. One other purpose of the current investigation was to determine the role of external melatonin treatment and internal melatonin release on liver antioxidant enzymes in single circadian rhythm.

MATERIALS AND METHODS

Chemicals

Xanthin, xanthin oxidase, NADH, reduced nicotinamide adenine dinucleotide phosphate (NADPH), superoxide dismutase (from bovine erythrocytes), Folin Ciocalteu reagent, cytocrome c (horse heart), ethylenediaminetetraacetic acid (EDTA), and BSA were obtained from Sigma Chemical Co., USA. Melatonin was obtained from Acros Organics, Belgium. H_2O_2, KH_2PO_4, Na_2HPO_4, $CuSO_4$, NaOH, Na_2CO_3 were obtained from Merck, Germany.

Animals

Six week old male inbred Swiss albino mice (25-30g) were used. Animals were maintained in a temperature controlled room (23˚C±2) under a photoperiod of 14 hour light and 10 hour dark, where the dark cycle period was from 8:00 PM to 6:00 AM. Mice were fed with pelleted standard laboratory mice feed (Terme Mice Food Ltd., Turkey) and tap water ad libitum.

Experimental procedure

In total 82 Swiss albino mice were used, divided into two main groups: (I) control group and (II) melatonin-treated group. A single dose of melatonin 10 mg/kg body weight injected intraperitonally as a pharmacological dose to melatonin treated group. Melatonin was dissolved in saline in <0.5% ethanol. Each of these groups were divided into six time-depended subgroups of 6-7 animals (1) 0 h, (2) 2 h, (3) 4 hour, (4) 8 hour, (5) 12 hour and (6) 24 hour. Twenty-four hours before the experiment, the mice were starved and allowed access to water ad libitum. Melatonin injections were given at 6:00 PM (time point 0). The control group was injected with the same amount of solution without melatonin. Depending on the group, mice were killed at 0 hour, 2 hour, 4 hour, 8 hour, 12 hour and 24 hour post injection by cervical dislocation and the liver was rapidly removed and processed as described below.

Preparation of mice liver homogenates

The liver was quickly perfused with ice-cold 0.9% saline to eliminate any possible effects due to diurnal variation. Mice livers were cut into small pieces and washed extensively with 0.15 M NaCl. Homogenates (w/v) were prepared in a sucrose solution (0.25 M sucrose, 10 mM Tris-HCl pH 7.4), using a homogenizer. Freeze thawing and ultrasonification for four times in 30 second for each application were applied of the specimens. The homogenates were centrifuged at 10000 g for 1 hour to remove debris. The clear supernatants were collected for enzymatic assays and the protein concentrations of the specimens were determined just before the analytic experiments by Lowry method using bovine serum albumin as standard at 600 nm [30]. Liver specimens were kept in the dark. All procedures were performed at 4˚C.

Determination of superoxide dismutase activity

Measurement of SOD activity was carried out spectrophotometrically [31]. The reaction mixture consisted of 50 mM potassium-phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.6 mM xanthine, 0.24 U/ml xanthine oxidase, 0.03 mM cytochrome c, 0.1 mM EDTA and an appropriate amount of the cytosol sample in a total volume of 3.0 ml. SOD was used as a standard. Superoxide radicals were detected by monitoring the reduction of cytochrome c at 550 nm. One unit of SOD is expressed as the amount required causing a 50% inhibition of the cytochrome c reduction.

Determination of catalase activity

The CAT activity was measured by monitoring its ability to degrade H_2O_2 in a spectrophotometrical assay at 240 nm [32]. The reaction mixture consisted of 50 mM potassium-phosphate buffer (pH 7.0), 30 mM H_2O_2 and an appropriate amount of cytosolic sample in a total volume of 3.0 ml. The units of catalase activity were calculated from the extinction coefficient of H_2O_2 at 240 nm (ε=39.4 M−1 cm−1). The activity is expressed as Units/mg cytosolic protein, where one unit is defined as the amount of liver cytosolic or fractionated protein, in mg, required to decompose 1.0 μmol of H_2O_2 per min at pH 7.0 at 25°C.

Statistical analyses

The data are expressed as the arithmetic mean ± S.D. of the number of animals (n); when P<0.01, the difference was considered to be statistically significant. All data are evaluated based on two main effects being groups that are compared as control versus melatonin treated and post injection lag. Statistical analysis of the data for multiple comparisons was performed by two-way analysis of variance (ANOVA). The significance of differences between means was determined by the t-test. Statistical analysis of the data was performed by using SPSS Version 12.0 (SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

Antioxidant enzymes are part of the primary cellular defense against free radicals induced by endogenous or exogenous factors in cells. Scientists conducted number of experiments to solve the underlying mechanism of the free radicals effects and pathogenesis. In both in vitro and in vivo experiments, melatonin has been found to protect different type of cells and tissues against oxidative damage induced by a variety of free-radical-generating drugs, like the carcinogens, carbon tetrachloride, ischemia-reperfusion, and ionizing radiation [33-36]. This study mainly focuses on the effect of melatonin on liver antioxidant status in a single circadian rhythm with time dependent manner with using inbred Swiss albino mice as a model system. We measured the effects of melatonin on antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT)

The first significant alterations determined in SOD level after 4 hour post injection of melatonin. Melatonin injection resulted with inhibition starting from 4 h to 24 h p.i. with descending nature by the time. However, in control specimens belong to dark cycle period of the experiment has higher SOD levels than light cycle of circadian period. The maximum inhibition of SOD was observed at 4 h p.i. (1.83 fold). It is clearly seen in the control group that SOD activity starts to increase at the beginning of the dark cycle of circadian rhythm (4-12 hour p.i. periods). These results revealed a significant (p<0.01) increase in dark cycle specimen (4, 8 and 12 h p.i.) relative to light cycle (0, 2 and 24 h p.i.) specimens. This clearly shows that internal melatonin release ameliorates the activity of SOD (Fig 1.). It has already known that SOD enzyme level was significantly higher in dark cycle period of light dark cycle than control SOD levels and melatonin increases the superoxide dismutase gene expression (13, 37-38). Our results clearly demonstrate that stimulatory effects of both exogenously administered melatonin and internal expression increases the levels of superoxide dismutase in liver tissue.

Administration of melatonin significantly (p<0.01) inhibited the CAT activity from 4 h to 24 h p.i. The inhibition of CAT started after 4 h p.i. and reached its maximum level at 8 h p.i. (the decrease was 3.56 times). After 8 h p.i. CAT inhibition slowed down (Fig 2.). Starting of light cycle period, it turned back to the control level (0 h p.i). It was inferred from the inhibition of the CAT that melatonin may directly scavenged H_2O_2. So, the direct inhibition can result in reduction of catalase activity. The decreased manner of the CAT inhibition may be attributed to external treatment of melatonin. In the course of time, it was metabolized and may be inactivated by light in the latter time points of the experiment. So catalase inhibition may be affected by either circadian rhythmic release of the melatonin or by external melatonin treatment. It has been known that melatonin scavenges H_2O_2 in pure chemical system with dose dependent manner. Direct reaction between melatonin and H_2O_2 resulting formation of intermediates that scavenges $\bullet O_2$ radicals [4, 5, 19]. It is already known that physiological levels of melatonin in a unicellular organism (Gonyaulax polyedra) prevent the lethal oxidative stress induced by H_2O_2 [10, 39]. So, we may explain SOD inhibition by intermediate interaction between $\bullet O_2$ radicals.

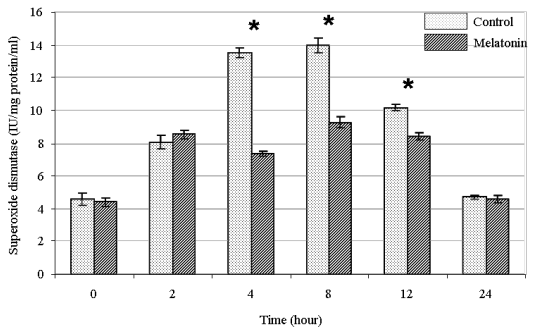

Fig 1. Changes in superoxide dismutase level in liver homogenates of control versus melatonin treated groups of Swiss albino mice in single circadian rhythm. SOD activity expressed as international unit/milligram protein/milliliter (IU/mg protein/ml). The values are the arithmetic means ± SDs. *p<0.01 was considered significant.

Furthermore, CAT inhibition by direct scavenging effect of the melatonin. Due to this dual effect of melatonin, it is considered as an important antioxidant in protecting the liver from oxidative stress. It was concluded from the results that melatonin has great inhibition effect on SOD and CAT level at dark cycle period of the circadian period in mice liver. In agreement with our results, Illnerova et al. [40] showed that pineal melatonin concentrations are higher in dark cycle than light cycle of circadian rhythm in Hamsters.

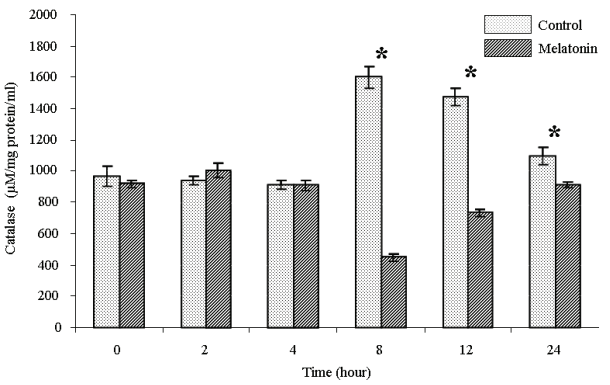

Fig 2. Changes in catalase level in liver homogenates of control versus melatonin treated groups of Swiss albino mice in single circadian rhythm. CAT activity expressed as micromoles/milligram protein/milliliter (µM/mg protein/ml). The values are the arithmetic means ± SDs. * p<0.01 was considered significant.

We therefore conclude that melatonin plays an important role in providing direct or indirect protection against free radical damage by stimulating antioxidant enzymes. In the current experiment, the protective effect of melatonin are related to free radical scavenging activity on liver which is related to increased SOD level or direct scavenging activity. Based on the data obtained from the control group, internal melatonin release increases SOD and CAT activities. External melatonin treatment may contribute direct inhibition of hydroxyl radicals as well as $\bullet O_2$ and stimulates antioxidant enzymes.

Consequently, melatonin could be considered as a potential therapeutic agent in liver pathologies where excessive free radical production has been implicated. Those results are in accordance with the findings of the antioxidant properties of melatonin so far.

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