Influence of subacute melatonin treatment on antioxidant factors in the liver of female rats

Mohammed Fathi Abdallah¹, Elif Karacaoglu², Gözde Girgin¹, Bilge Kilicarslan¹, Güldeniz Selmanoglu², Terken Baydar¹,*

¹Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Hacettepe University, Sihhiye Campus, Ankara, Turkey; ²Department of Biology, Faculty of Science, Hacettepe University, Beytepe Campus, Ankara, Turkey

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ABSTRACT

Melatonin is a well-known antioxidant substance. The attention paid to its adverse effects is limited. Moreover, data published about its toxicity and/or undesired effects are scarce in the literature. The main aim of the present study was to evaluate subacute melatonin effects on antioxidant enzymes, lipid peroxidation and liver transferases in experimental animals. Female Wistar rats were used in this study and divided into two groups. Group I was a control group and received corn oil. Group II was treated with 100 mg/kg (oral) melatonin for 7 days. The antioxidant enzyme activities such as superoxide dismutase (SOD), catalase (CAT) and lipid peroxidation marker malondialdehyde (MDA) levels were measured. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), cholesterol and urea levels were also measured. Additionally, histopathological evaluations of hepatic tissue samples were performed. The obtained results showed that the subacute melatonin exposure reduced both antioxidant CAT activity and MDA levels (both, p<0.05). However, ALT and AST levels increased in melatonin treated rats (both, p<0.05). The ratio of AST to ALT in the melatonin treated group was nearly two-fold higher than the control group. Microscopic examination revealed no severe histopathological changes in the liver. In conclusion, melatonin treatment causes changes in female rats liver functions and further investigations are necessary to examine the full impact of melatonin supplementation with regards to route of administration, dose as well as the exposure period.

KEYWORDS: Catalase, histopathology, malondialdehyde, melatonin, transaminases.
INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine) is an indolamine secreted mainly by the pineal gland particularly during the dark hours of the night as well as many other peripheral tissues and organs (Lerner et al., 1958; Acuña-Castroviejo et al., 2014). Melatonin has a potent antioxidant activity against both reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Iriti et al., 2010). The protective effects of melatonin against toxic free radicals have been described in several experimental and clinical reports (Rodríguez et al., 2007; Galano et al., 2013; Zhang and Zhang et al., 2014; Abdel Moneim et al., 2015).

The direct scavenging actions of melatonin as well as its indirect effects on the enzymes which inhibit the synthesis and/or detoxify the oxidative stress inducers such as hydroxyl radical, hydrogen peroxide, peroxynitrite anion, nitric oxide and hypochlorous acid are induced with both physiological and pharmacological levels of melatonin (Reiter et al., 1995; 1997; Kolli et al., 2013; Ma et al., 2014).

The present study was undertaken to evaluate the possible effects of subacute exposure to a high dose melatonin on liver antioxidant status and hepatic biochemical parameters in female rats. For this purpose, two major antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT) were evaluated. In addition, malondialdehyde (MDA) as a lipid peroxidation marker, hepatic function biomarkers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activities, and levels of cholesterol and urea were evaluated. Furthermore, hepatic histopathological examination was performed to all liver tissues.

MATERIALS AND METHODS

Animals

A total of 10 female Wistar rats with body weights (b.w.) of 174–213 g were obtained from Experimental Animals Unit, Hacettepe University.

Diet and Chemicals

All chemicals used in the study were of analytical grade. They were obtained from Sigma Chemical Co and used as purchased without any further purification. The rat chow contained crude protein not less than 23%, ash not more than 8%, crude fiber not more than 7%, salt (1%), moisture not more than 12%, and metabolizable energy, contents minimum 2.5 kcal/gram.

Experimental protocol

The animals were randomly divided into 2 groups and all the performed treatments were applied for 7 days. Group I (n=5) was used as a control group while Group II (n=5) was treated with 100 mg/kg (b.w.) dose of melatonin orally. Changes in the body weights of each animal were observed once per day during the experiment period.

All the animals were kept under the same laboratory conditions. They were housed as 5 rats per a cage, at 22-24°C and 50±10% humidity with a 12:12-hrs light-dark cycle and they had access to feed and tap water ad libitum. The study was performed with the official permission of the “The University Ethical Committee for the Protection of Animals in Research” (#2008/15).

Sampling

At the end of the experimental period (day 8), all rats were sacrificed under anesthesia using Ketalar® (Pfizer, Turkey) and Alfazyne® (Alfasan International, Holland). Liver, kidney and heart samples were immedi-
ately removed, and absolute organs weight were recorded. Relative liver, kidney, and heart weights were calculated based on organ-to-body weight ratio.

**Determination of hepatic functions**

In order to assess the hepatic functions, the blood samples were collected and centrifuged for 15 min at 3000 rpm (Hanil Science Industrials Co.). The obtained serum samples were stored at -20°C until the analysis of urea, total cholesterol and the enzyme activities, AST, ALT and ALP. Determination of these hepatic enzyme activities, cholesterol and urea levels were done by using biochemical kits (Audit Diagnostics, Ireland) and Shimadzu CL-770 Clinical Spectrophotometer (Japan).

**Histopathological evaluation**

Briefly, before storing at -80°C till analysis, each liver tissue sample was fixed in 10% formalin solution, processed in the routine histological processes and tissue sections were prepared in 5μm thickness and stained with haematoxylin and eosin (H&E). Hepatic tissues were examined histopathologically using Olympus BX51 system light microscope.

**Determination of specific SOD and CAT activities**

*Superoxide dismutase measurement*

Each liver homogenate was diluted with 50 mM Tris-HCl buffer which contains 1.2 mM EDTA (pH=8.2). 120 μl of diluted homogenate was mixed with 2800 μl of buffer solution. Subsequently, 120 μl of 6 mM pyrogallol solution in 10 mM HCl was added to the mixture and mixed for 30 sec. The decrease in absorbance was followed at 420 nm for 2 min by Shimadzu UV-1700 PharmaSpec spectrophotometer (Japan). Non-enzymatic reaction rate as blank was determined by substituting buffer solution for the sample and this reaction rate was used for the activity measurements.

*Catalase measurement*

Each tissue homogenate was diluted with 50 mM phosphate buffer at pH=7.00, just before the measurements. The reduction rate of 10 mM hydrogen peroxide was followed at 240 nm for 30 sec at room temperature (Shimadzu UV-1700 PharmaSpec Spectrophotometer).

*Total protein determination*

The protein content of the lysates was determined according to the method of Lowry modified by Miller (1959) using bovine serum albumin as standard (Miller, 1959).

**Determination of the lipid peroxidation products**

The liver homogenates were used for estimation of MDA levels by using the method based on thiobarbituric acid (TBA) reactivity (Rungby and Ernst, 1992) with tetraethoxypropane as a standard (Narang et al., 2004).

**Statistical analysis**

The obtained data were analyzed using SPSS version 11.0 (SPSS Inc., Chicago, IL). The homogeneity of variance and normal distribution was evaluated using general linear model procedure and Kolmogorov-Smirnov nonparametric test. Body and relative organ weights were examined using one-way ANOVA. Comparisons between the two groups were detected using Mann Whitney U Test. Statistical significance was set at p<0.05.
RESULTS

The basic toxic response indicators, death and body weight changes were investigated in the two study groups. There was no mortality observed in both groups during the experimental period. Changes in the body weight gains are shown in Figure 1, and there were not any significant differences in body weight gain of the two groups. It is known that organ weight changes have been accepted as an indicator of chemically induced changes. The comparisons with relative major organ weights of the treated and untreated groups are given in Table 1. The only significant alteration was observed in liver weights; melatonin caused a decrease in relative liver weights in comparison with the control group (p<0.05).

![Graph showing changes in body weight over time for Control Group I and Melatonin Group](image-url)

**Figure 1**: Changes in body weights during the experiment as means ± SEM.

The results of liver CAT enzyme activities are shown in Figure 2. Compared to the control, a significant decrease in CAT enzyme activity was detected in melatonin treated group (p<0.05). However, as shown in Figure 3, no difference in SOD activity was observed between melatonin exposed and non-exposed groups (p>0.05).

**Table 1**: Relative organ weights of the groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Relative organ weights (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>Control</td>
<td>0.089 ± 0.002</td>
</tr>
<tr>
<td>Melatonin</td>
<td>0.040 ± 0.001*</td>
</tr>
</tbody>
</table>

Values are means± SEM. *p<0.05 versus control rats.
Figure 2: Effect of melatonin on specific catalase (CAT) activity (IU/mg protein) in rat liver. *p<0.05 versus control rats.

Figure 3: Effect of melatonin on specific superoxide dismutase (SOD) activity (IU/mg protein) in rat liver.

Levels of MDA, cholesterol and urea are presented in Table 2. MDA levels decreased in melatonin treated group in comparison with the control, and there was a statistical significance between the two groups (p<0.05). Melatonin caused a significant decrease in urea levels (p<0.05), while the cholesterol levels were not affected (p>0.05).
Table 2: Results of malondialdehyde (MDA), cholesterol and urea measurements.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Parameters</th>
<th>MDA (nmol/g tissue)</th>
<th>Cholesterol (mg/dL)</th>
<th>Urea (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.215 ± 0.031</td>
<td>61.42 ± 4.18</td>
<td>66.12 ± 3.94</td>
</tr>
<tr>
<td>Melatonin</td>
<td></td>
<td>0.124 ± 0.010*</td>
<td>48.98 ± 3.93</td>
<td>45.28 ± 2.74*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. *p<0.05 versus control rats.

As shown in Table 3, melatonin significantly increased AST and ALT levels in melatonin group compared to control (both, p<0.05). The ratio of AST to ALT in melatonin treated group was nearly two-fold higher than the control group (Table 3).

Table 3: Activities of liver function enzymes and the ratio AST to ALT in study groups.

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>Groups</th>
<th>Control</th>
<th>Melatonin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td></td>
<td>18.50 ± 0.95</td>
<td>24.86 ± 1.93*</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td></td>
<td>80.46 ± 2.98</td>
<td>197.82 ± 27.83*</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td></td>
<td>73.60 ± 5.37</td>
<td>128.86 ± 29.52</td>
</tr>
<tr>
<td>AST/ALT</td>
<td></td>
<td>4.35 ± 3.14</td>
<td>7.96 ± 14.42</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. *p<0.05 versus control rats.

In histological examination, there were minimal changes such as mononuclear cell infiltration and cytoplasmic degeneration in only one liver of the five rats in Group II. The minor alterations are presented in Figure 4.

**DISCUSSION**

Melatonin acts as a direct scavenger by inducing the expression of antioxidant enzymes such as glutathione peroxidase, glutathione reductase, and superoxide dismutase. These actions further reduce the oxidation state in the cells, and/or improve mitochondrial homeostasis (Rodriguez et al., 2004; Zhang and Zhang et al., 2014). In the present study, substantial changes in the activity of CAT enzyme were observed, but there was no effect on the SOD activity. This finding may be speculated as antioxidant properties of melatonin followed no pattern on subacute exposure. Reduced lipid peroxidation may suggest that melatonin exerts its antioxidant effect through radical scavenging activity rather than its restoring activity on antioxidant defense. However, with contrast to all reported findings about the protective effects of melatonin supplementation, data about its adverse effects is scarce.
**Figure 4:** Photomicrographs of liver tissues of rats in control and melatonin groups stained with H&E. **A**: Control group and **B**: Melatonin group, mononuclear infiltration (arrow), cytoplasmic degeneration (arrow head) (200x).
A few animal studies reported that supplementations of high doses of exogenously administered melatonin during pregnancy can have clear adverse effects on body weight for both mother and pups (Singh et al., 2013). Other studies claimed that melatonin may cause delayed sexual maturation, infertility, hypothermia, disturbance in blood pressure regulation and reduced body weight gain (Sorrentino, 1975; Lang et al., 1984; Graham et al., 2004; Singh et al., 2012). The explanation for the conflicting findings is unclear, but it might be associated with melatonin dose and the duration.

It is reported that melatonin levels reach saturation in the nucleus and mitochondria with a dose of 40 mg/kg (b.w.). However, parenteral melatonin at higher doses did not cause further accumulation of melatonin in these organelles (Venegas et al., 2012). In some previous reports, melatonin dose for sub-chronic and chronic treatment ranged between 2 and 10 mg/kg (b.w.) (Turgut et al., 2013; Othman et al., 2014; Shokrzadeh et al., 2014; Abdel Moneim et al., 2015). Jahnke et al. (1999) documented that the maternal toxicity can be observed at 200 mg/kg where a clear reduction in food intake and body weight gain were recorded. Furthermore, the developmental toxicity of melatonin starts from 100 mg/kg.

The ability of melatonin to protect different organs as liver, heart and kidney against free radical damage are well documented in vitro and in vivo (Deng et al., 2006; Rodríguez et al., 2007; Bharti et al., 2014). Alterations in liver function enzymes such as ALT, AST and ALP showed the biochemical toxicity.

There are a few studies on non-invasive assessment of fibrosis, cirrhosis and liver-related death by the ratio AST to ALT. In these cases, it is suggested that increased AST/ALT is associated with liver cirrhosis regardless the cause. Even though the reason for the increased ratio is unknown, it is claimed that the sinusoidal clearance of AST decreases in cirrhotic circumstances (Nyblom et al., 2006; Alempijevic et al, 2009; Wedemeyer et al., 2010). In here, the mean AST/ALT value of melatonin exposed rats was nearly two-fold higher than control. Solely, serious hepatotoxic effect of melatonin cannot be attributed; since signs of histopathology did not support the biochemical changes.

In conclusion, the current findings of the study may have important implications in melatonin applications at 100 mg/kg subacute dose. Indeed, further investigations are highly recommended to examine the full impact of melatonin supplementation in terms of dose and/or duration. We believe that future studies will be able to explain the efficacy and the safety of melatonin.

CONFLICT OF INTERESTS
The authors have declared no conflict of interests.

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Corresponding author:
Prof. Dr. Terken Baydar
Department of Pharmaceutical Toxicology, Hacettepe University Faculty of Pharmacy, Sihhiye, 90-06100, Ankara, Turkey
Tel: +90 (312) 3092958
Fax: +90 (312) 3114777
E-mail: tbaydar@hacettepe.edu.tr