Effects of pinealectomy and exogenous melatonin on the brains, testes, duodena and stomachs of rats

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Abstract. – BACKGROUND, It is generally agreed that physiological levels of melatonin, a hormone secreted by the pineal gland, are important in protecting against oxidative stress-induced tissue damage.

AIM, We investigated the effects that pinealectomy and the administration of exogenous melatonin have on the brains, testes, duodena and stomachs of rats.

MATERIALS AND METHODS, Pinealectomized (Px) and sham-operated (non-Px) rats were used. We evaluated structural changes, and catalase (CAT), reduced glutathione (GSH), super oxide dismutase (SOD) and malondialdehyde (MDA) levels. The rats were divided into the following five groups (eight rats in each group): sham (non-Px), Px+ vehicle, Px+ melatonin (10 mg/kg given daily intraperitoneally for a week), melatonin and ethyl alcohol.

RESULTS, The antioxidant levels in the tissue of Px rats were significantly lower than in those of the sham group. Administering melatonin significantly increased antioxidant levels (p < 0.05). The Px rats also showed a significant increase in MDA levels when compared to the sham group, and administering melatonin to the Px rats significantly reduced their MDA levels (p < 0.05). The severity of caspase-3 staining was lower in the Px+ melatonin group than in the Px+vehicle group.

CONCLUSIONS, These findings suggest that significantly more oxidative and structural changes occur in rats’ brains, spinal cords and testes after pinealectomy, but that this can be diminished by melatonin treatment. However, Px does not have important effects on the duodenum and stomach.

Key Words:
Pinealectomy, Melatonin, Brain, Testis, Duodenum, Stomach.

Introduction

The pineal gland was identified a long time ago, and considerable research has been done on this gland and its main product, melatonin. But, how the pineal gland functions in humans is still poorly understood. The synthesis of melatonin is regulated by the environmental light/dark cycle, through the suprachiasmatic nucleus, and melatonin is mainly secreted at night. However, night-time melatonin production declines progressively with age. Melatonin is known to be a potent free radical scavenger and antioxidant. It plays an important role in various physiological processes, including modulation of circadian rhythms, reproductive physiology, sleep, body temperature, aging and stimulation of immune functions. It also has antiapoptotic, oncostatic, neuro-protective and cardio-protective effects.

The decrease with age in the total antioxidant capacity of human serum is closely correlated with its melatonin concentration. In accordance with the free radical theory of aging, antioxidant enzymes and molecule activity decrease with age. Thus, reduced production of melatonin, accumulated free radicals and disturbance in the pro-oxidant/antioxidant balance may be responsible for the senescence of organisms and the development of age-related degenerative diseases. In addition, free radicals can react with DNA, RNA, proteins and membrane lipids, and lead to cellular toxicity. In recent years, many investigators have suggested that antioxidants have a useful effect on a large number of age-related diseases, such as atherosclerosis, Alzheimer’s disease, osteoporosis.

Melatonin scavenges both hydroxyl radicals and peroxyl radicals, and it directly neutralizes a
number of free radicals and reactive oxygen species (ROS). It also stimulates several antioxidative enzymes such as super oxide dismutase (SOD), glutathione (GSH) reductase, catalase (CAT)\textsuperscript{12,13}. Reiter et al\textsuperscript{14} showed that pinealectomy (Px) leads to reduction in the physiological levels of melatonin. This decrease exacerbates oxidative damage and administering exogenous melatonin can reduce the impact of injury\textsuperscript{14,15}.

In this study, we investigated the effects of physiological and pharmacological levels of melatonin on rat brains, testes and gastrointestinal tracts, using pinealectomy (Px) and exogenous melatonin. This article provides extra evidence that a melatonin deficiency may be responsible for age-related changes in the brain, testis and gastrointestinal tract and that melatonin has curative effects in reducing these changes.

**Materials and Methods**

**Animals**

Forty male Wistar rats, weighing 150-200 g were placed in a quiet room, with controlled temperatures (21 ± 2°C) and humidity (60 ± 5%), and a 12-12-hour light-dark cycle. The rats had free access to both food and water. The experiment was performed in accordance with the National Institute of Health guidelines for animal research and was approved by the Committee on Animal Research, at Inonu University, Malatya. The rats were Px or non-Px six months before the beginning of any injections.

**Pinealectomy**

Pinealectomy was performed as described by Kuszak and Rodin\textsuperscript{16}. The rats were anesthetized with ketamine hydrochloride (75 mg/kg) and xylazine (8 mg/kg) before the operation. The procedure was completed within 15 minutes. Pinealectomy was confirmed through histological evaluation of the gland for each animal.

**Experimental Groups**

Px and non-Px rats were randomly divided into five groups, each containing eight rats as follows:

**Group I (Sham):** Non-Px

**Group II (Px+vehicle):** Px rats were followed for six months. Vehicle was administrated daily, intraperitoneally (i.p.) by injection, for a week.

**Group III (Px + MEL):** Melatonin (10 mg/kg) was administrated daily as an i.p injection to the Px rats for a week. Melatonin (Sigma, St. Louis, MO, USA) was dissolved in ethanol and then diluted in saline (the final concentration of ethanol was 1%). All injections were administered at 17:00 hours.

**Group IV (MEL):** Melatonin (10 mg/kg) was administrated daily as an i.p injection for a week.

**Group V (Px + E):** Animals were treated with 1% ethanol.

After the last injection, all rats were sacrificed and their brains, spinal cords, testes, stomachs and parts of the small intestines (duodena) were quickly removed and divided into two parts. One part was put in a formaldehyde solution for histopathological examination by light microscopy. The other half of the tissues were placed in liquid nitrogen and stored at −70°C until assayed for levels of malondialdehyde (MDA), SOD, GSH, CAT.

**Biochemical Determination**

Blood was drawn from the vena cava inferior to determine the levels of MDA (end product of lipid peroxidation), GSH-Px, GSH, SOD and CAT. The serum was separated by centrifugation (1,000 rpm, 10 min) at 4°C and was stored at −80°C until the biochemical parameters were determined.

The MDA content of serum was determined spectrophotometrically (A Shimadzu 1601 UV/VIS spectrophotometer, Shimadzu, Kyoto, Japan), by measuring the presence of thiobarbituric acid reactive substances (TBARS)\textsuperscript{17}. Results are expressed as nmol/g tissue.

CAT activity was determined according to the Aebi method\textsuperscript{18}. Briefly, 10 ml of supernatant tissue were added to 2.99 ml of phosphate-buffered saline (PBS) and the absorbance was read at 240 nm, using a UV spectrophotometer. The principle of the assay is based on the determination of the rate constant (s) or the H$_2$O$_2$ decomposition rate at 240 nm. Results were expressed as k (rate constant)/mg of protein.

Determining SOD enzyme activity was based on the production of H$_2$O$_2$ from xanthine-by-xanthine oxidase and reduction of nitroblue tetrazolium\textsuperscript{19}. The product was evaluated spectrophotometrically at 560 nm. Results are expressed as U/mg protein.
GS H was determined by a spectrophotometric method, based on the use of Ellman’s reagent\textsuperscript{20}. Tissue homogenates were mixed with 50% trichloroacetic acid in distilled water in glass tubes and centrifuged at 3,000 rpm for 15 minutes. The supernatants were mixed with 0.4M Tris buffer, pH 8.9, and 0.01M 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) was added. After shaking the reaction mixture, its absorbance was measured at 412 nm. The absorbance values were expressed as µmol/g tissue.

**Histological Examination**

Histopathological and immunohistochemical analyses were performed in all cases, on formalin-fixed and paraffin-embedded tissues. Sections of 5-mm thickness were cut, deparaffinized, hydrated and stained with caspase 3, and the apoptotic cells (hypoxic neurons) were assessed. Sections on poly-L-lysine-coated slides were used after drying in an oven for 1 hour at 60 C. The sections were dewaxed in xylene, rehydrated in ethanol and incubated for 8 minutes in 3% hydrogen peroxide to block endogenous peroxidase. After washing in PBS, the sections were incubated for 5 minutes in ultraviolet block. Each slide was then incubated for 30 minutes at room temperature with the primary polyclonal rabbit anti-mouse antibody to caspase 3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at appropriate dilutions (1:100). After washing in PBS, the slides were incubated with large-volume streptavidin peroxidase for 13 minutes. Finally, the preparations were developed in AEC (3-Amino-9-EthylCarbazole) chromogen, counterstained with hematoxylin and mounted with Aqueous-Mount. Biopsies were evaluated at x400 magnification by a minimum of 10 fields for each brain, spinal cord and testis slide. A score from 0 to 3 was given for hypoxic neurons and cells of seminifer tubuli profiles involving an intersection as follows: grade 0, normal histology; grade 1, mild injury consisting of red neurons; grade 2, moderate, as for score 1, but greater than one-third and less than two-thirds of hypoxic neurons; grade 3, severe hypoxic neurons.

**Statistical Analysis**

Data were analyzed statistically using the SPSS statistical program (SPSS Inc., Chicago, IL, USA). The results were statistically analyzed by one-way ANOVA. Post hoc comparisons were performed using the Tukey method. Differences were considered significant when $p < 0.05$. All results are expressed as mean ± SEM.

**Results**

**Histological Results**

We evaluated the apoptosis in the seminiferous tubules, cerebellum Purkinje cells, cortical neurons and brain stem cells, and in the medulla spinalis grisea region (Table I). Mild staining was observed in the sham group and we interpreted this to be a normal process of spermatogenesis and aging. The Px group had intense caspase-3 staining (Figure 1A, C, E, 2A) in all of the cells except for those in the duodenum and stomach. The duodenum and stomach showed normal histology. Also, administering melatonin to the Px rats decreased apoptosis, and the Px+MEL group had mild staining (Figure 1B, D, F, 2B). Staining for the MEL group was similar to that of the sham group. Ethyl alcohol showed no effects on the cells.

**Biochemical Results**

There was no significant difference in the MDA and antioxidant levels in duodenum and stomach tissues. MDA and antioxidant levels in brain and testis tissues are summarized in Table II and Table III. Px caused a significant increase in MDA levels, compared to those of the sham (non-Px) group. When melatonin was given to Px rats, they had considerably reduced MDA levels in their tissues. SOD, CAT and GSH levels were significantly lower in the Px group than in the control group. Administering melatonin significantly increased these levels.

**Discussion**

Melatonin is synthesized in the pineal gland, from the neurotransmitter serotonin, and acts as a direct free radical scavenger and also an indirect antioxidant\textsuperscript{21,22}. In humans, the total antioxidative capacity of serum is related to melatonin levels. These levels reduce with age. The central nervous system is one of the major target organs for oxidative stress because of its vulnerability to damage from free radicals, its relatively weak antioxidative defense system and its inability to regenerate. The brain uses a lot of oxygen (20% of the total oxygen inspired) and has a high content of iron, which is involved in generating the hydroxyl radical\textsuperscript{23,24}. Therefore, deterioration in the central nervous system may be especially important in aging. Neurodegenerative changes and dementia are major and debilitating features of aging\textsuperscript{25}. It has been suggested that melatonin plays an im-
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Table I. Apoptosis grade in cortical neurons, purkinje cells, spinal cord, brain stem neurons, and testes.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Apoptosis (caspase-3)</th>
<th>Purkinje cells n=8</th>
<th>Cortical neurons n=8</th>
<th>Brainstem neurons n=8</th>
<th>Spinal cord n=8</th>
<th>Seminifer tubul n=8</th>
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<tbody>
<tr>
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<td>5</td>
<td>7</td>
<td>6</td>
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<td>5</td>
</tr>
<tr>
<td></td>
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<td>3</td>
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<td></td>
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<td>7</td>
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Important role in the pathogenesis of age-related neurodegenerative diseases. There are a variety of endogenous and exogenous antioxidants, such as tocopherol (vitamin E), ascorbic acid (vitamin C) and beta carotene, that reduce free radical injury and protect neuronal and glial integrity. Research has looked at the antioxidant effects of melatonin, which is known as the most powerful antioxidant. It has been shown to be very effective, especially in the brain. This high efficacy may relate to the fact that melatonin can pass easily through the blood-brain barrier and that there are higher levels of it in the cerebrospinal fluid than in the blood. In this study, we demonstrated that significant neuron damage (oxidative damage and structural changes) results in Px rats compared to rats in the sham group and that tissue damage in Px rats is prevented by administering exogenous melatonin. This protection was exhibited through reduced levels of lipid peroxidation products, increased levels of antioxidant and less apoptosis in the cells.

Like other organ systems, the male reproductive organs undergo a variety of age-related changes, such as reduced sperm output, more abnormal forms of sperm, reduction in size and endocrine dysfunction. The causes of cellular dysfunction in the testes during aging are unknown, but oxidative stress is implicated. Spermatogenesis is an extremely active replicative process and requires high rates of mitochondrial oxygen consumption by the germinal epithelium. Thus, both spermatogenesis and Leydig cell steroidogenesis are vulnerable to oxidative stress. Awad et al. reported that serum and seminal plasma melatonin levels in all infertile groups were less than among fertile groups. Also, it has been determined that the administration of antioxidants such as caffeic acid phenethyl ester, licopene, beta carotene and resveratrol have protective effects against oxidative stress created in the testes through alternative mechanisms. Melatonin is one of the most effective antioxidants in protecting testicular function. Melatonin can readily cross all physiological barriers, such as the blood-testes barrier, and enter every cell and all subcellular compartments. Our investigation demonstrated morphometric and biochemical changes in Px rat testes. Px induced an increase in apoptotic seminiferous tubules and MDA levels and decreased antioxidants such as CAT, SOD and GSH. Administering exogenous melatonin can prevent damage to the testes in Px rats.

Melatonin is a hormone with endocrine, paracrine and autocrine actions that is synthesized by the gut, bone marrow cells, lympho-
cytes, mast cells and epithelial cells, as well as by the pineal gland. It is believed that melatonin produced in the gut acts both as a paracrine molecule and as a hormone released into the portal vein. Release of melatonin from all these extra-pineal sources seems to be independent of the photoperiod. The fact that at any time of the day or night the gut contains at least 400 times more melatonin than the pineal gland further emphasizes the functional importance of melatonin in

Figure 1. Histology of brainstem cell, purkinje cell and spinal cord of Px and Px+M groups after caspase-3 staining. (A) Px, brainstem cell. (B) Px+M, brainstem cell. (C) Px, spinal cord cell. (D) Px+M, spinal cord cell. (E) Px purkinje cell. (F) Px+M, purkinje cell, X200.

Figure 2. Px group (A); There is an intense caspase-3 staining in seminifer tubule. Px+M group (B); melatonin administration to Px rats decreased apoptosis, X400.
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Table II. Mean MDA, SOD, CAT and GSH values in testes tissue of the groups (mean values ± SEM).

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA (nmol/g tissue)</th>
<th>SOD (U/mg protein)</th>
<th>CAT (k/mg protein)</th>
<th>GSH (μmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>103.24 ± 3.81</td>
<td>3.68 ± 0.21</td>
<td>73.79 ± 1.47</td>
<td>111.21 ± 7.98</td>
</tr>
<tr>
<td>Px+Vehicle</td>
<td>184.78 ± 9.05a</td>
<td>3.33 ± 0.08a</td>
<td>38.16 ± 2.79a</td>
<td>71.64 ± 3.19a</td>
</tr>
<tr>
<td>Px+MEL</td>
<td>122.74 ± 13.19b</td>
<td>3.08 ± 0.1b</td>
<td>69.78 ± 8.27b</td>
<td>98.56 ± 6.52b</td>
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<tr>
<td>MEL</td>
<td>94.94 ± 43.15c</td>
<td>3.39 ± 0.12c</td>
<td>79.04 ± 6.60c</td>
<td>114.18 ± 5.46c</td>
</tr>
<tr>
<td>Px+E</td>
<td>112.49 ± 10.95c</td>
<td>3.24 ± 0.12c</td>
<td>68.06 ± 0.72c</td>
<td>99.07 ± 5.90c</td>
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</tbody>
</table>

Px: Pinealectomy; M: Melatonin; E: Ethyl alcohol. aIndicates a statistically significant difference compared with that of the sham group, (p < 0.05). bIndicates a statistically significant difference compared with that of the Px group, (p < 0.05). cNo significant when compared with sham group, (p > 0.05).

Table III. Biochemical results in brain tissue (mean values ± SEM).

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA (nmol/g tissue)</th>
<th>SOD (U/mg protein)</th>
<th>CAT (k/mg protein)</th>
<th>GSH (μmol/g tissue)</th>
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<tr>
<td>Sham</td>
<td>27.73 ± 2.39</td>
<td>15.75 ± 1.12</td>
<td>63.28 ± 2.29</td>
<td>2.95 ± 0.03</td>
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<tr>
<td>Px+Vehicle</td>
<td>41.63 ± 3.08a</td>
<td>6.77 ± 0.35a</td>
<td>42.82 ± 2.13a</td>
<td>2.13 ± 0.08a</td>
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<td>Px+MEL</td>
<td>25.50 ± 1.15b</td>
<td>11.42 ± 0.73b</td>
<td>55.58 ± 2.92b</td>
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</tr>
<tr>
<td>MEL</td>
<td>25.90 ± 1.79</td>
<td>10.85 ± 1.29</td>
<td>58.99 ± 3.57</td>
<td>2.71 ± 0.0</td>
</tr>
<tr>
<td>Px+E</td>
<td>26.81 ± 2.19</td>
<td>14.24 ± 1.37</td>
<td>65.11 ± 1.61</td>
<td>2.82 ± 0.0</td>
</tr>
</tbody>
</table>

aSignificantly different from sham group (p < 0.05); bSignificantly different from Px group (p < 0.05).

the gut. Messner et al. studied the distribution of melatonin in the human hepatobiliary-gastrointestinal tract and found high concentrations of melatonin in both gastric and duodenal mucosa. Our study confirmed the findings of other experimental studies, indicating that Px has no effect on the duodenum or the stomach.

The reduction of melatonin with age may be related to oxidative damage in the elderly. We showed that administering melatonin to Px rats has beneficial effects, as indicated by reduced levels of MDA and less morphological damage, and also enhances the antioxidative defense system. In addition, the biochemical and histological indicators in our investigation support the hypothesis that melatonin deficiency may play an important role in oxidative stress, and exogenous melatonin may reduce tissue injury that results from oxidant stress.

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