Radioprotective Effects of Melatonin on Radiation-Induced Cataract

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Gamma irradiation/Lens/Cataract/Melatonin/Antioxidant enzymes.

One of the mechanisms proposed to explain lens opacification is the oxidation of crystallins, either by radiation or reactive oxygen species (ROS). It has been shown that melatonin has both an anti-peroxidative effect on several tissues and a scavenger effect on ROS. The purpose of this study was to determine the antioxidant role of melatonin (5 mg/kg/day) against radiation-induced cataract in the lens after total-cranium irradiation of rats with a single dose of 5 Gy. Sprague-Dawley rats were divided into four groups. Control group received neither melatonin nor irradiation. Irradiated rats (IR) and melatonin+irradiated rats (IR+Mel) groups were exposed to total cranium irradiation of 5 Gy in a single dose by using a cobalt-60 teletherapy unit. IR+Mel and melatonin (Mel) groups were administered 5mg/kg melatonin daily by intraperitoneal injections during ten days. Chylack’s cataract classification was used in this study. At the end of the 10th day, the rats were killed and their eyes were enucleated to measure the antioxidant enzymes i.e. the activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), and lipid peroxidation level (malondialdehyde (MDA)). Irradiation significantly increased the MDA level, as an end product of lipid peroxidation, and also significantly decreased SOD and GSH-Px activity, emphasizing the generation of increased oxidative stress. Rats injected with melatonin only did not cause cataract formation. Melatonin supplementation with irradiation significantly increased the activity of SOD and GSH-Px enzymes and significantly decreased the MDA level. Total cranium irradiation of 5 Gy in a single dose enhanced cataract formation, and melatonin supplementation protected the lenses from radiation-induced cataract formation. Our results suggest that supplementing cancer patients with adjuvant therapy of melatonin may reduce patients suffering from toxic therapeutic regimens such as chemotherapy and/or radiotherapy and may provide an alleviation of the symptoms due to radiation-induced organ injuries.

INTRODUCTION

The destructive action of ionizing radiation is predominantly due to reactive oxygen species (ROS), including superoxide radical (O$_2^-$), hydroxyl radical (OH$^-$), and hydrogen peroxide (H$_2$O$_2$) generated by the decomposition of water.$^{1,1}$ Oxidative stress arises, when rates of ROS production outpace rates of removal. Oxidative stress is associated with various degenerative diseases, including cataract, macular degeneration, cancer, and arteriosclerosis.$^{5}$ Because of the serious damaging potential of ROS, cells depend on the elaboration of the antioxidant defence system (AODS), both enzymatic and non-enzymatic antioxidant defence mecha-

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nisms.$^{3}$ The important anti-oxidative enzymes are superoxide dismutases (SOD), catalase(CAT), glutathione peroxidase (GSH-Px). SOD enzyme catalyzes the dismutation of O$_2^-$ into H$_2$O$_2$. H$_2$O$_2$ can be transformed into H$_2$O and O$_2$ by CAT and GSH-Px enzymes. One of the indices of oxidative damage is the malondialdehyde (MDA) formation as an end product of lipid peroxidation.$^{4,5}$

Cataract is an opacity of the lens affecting vision.$^6$ The ocular lens is one of the most radiosensitive tissues and the lens epithelium is considered to be the initiation site for the development of radiation-induced cataract.$^7$ Radiation-induced cataract occurs depending on the increase of ROS generation, the destroy of epithelial DNA chains in the lens and the suppression of lens epithelial cell repair.$^8$ Merriam and Focht$^9$ reported that radiation induced cataract occurred a period over 3 months at the doses of 2 Gy given single and 5.5 Gy given fractioned.

Melatonin, synthesized from serotonin and realized in mammals during dark-phase of the cycle and endogenous antioxidant found in high concentrations in the pineal gland, was recently identified as a multifaceted direct free radical
scavenger as well as an indirect antioxidant when stimulating antioxidant enzymes. In addition, it has been demonstrated that melatonin was produced in many other tissues, including retina, lens. While pineal melatonin passes freely membranes and distributes in all body compartments, retinal melatonin is thought to act locally within the eye. Thus, it is possible that melatonin in the lens may have a function as a free radical scavenger within retinal photoreceptors, in the prevention of oxidative damage caused by ionizing radiation. The experimental evidences have supported that melatonin as an antioxidant agent protected cells both activating antioxidant enzymes in the reduction of free radicals production and stabilizing membranes against oxidative damage. To further assess this drug’s potential oxidative stress-preventive effects, these effects of melatonin were studied in rats by using a radiation-induced cataract model.

MATERIALS AND METHODS

Animals and experimental design

Forty albino female Sprague-Dawley rats, 8–12 weeks old, weighing $185 \pm 35$ gr the time of radiation bred at Ataturk University Medical School, Department of Pharmacology Experimental Animal Laboratory were used for the experiment. All animals received humane care in compliance with the guidelines of criteria of the Ataturk University Research Council. All procedures involving Sprague-Dawley rats adhered to the ARVO Resolution on the Use of Animals in Research. The rats were quarantined for at least one day before irradiation, housed ten to a cage which has the size of $60 \times 45 \times 30$ cm (width, length and height, respectively) in a windowless laboratory room with automatic temperature ($22 \pm 1^\circ$C) and lighting controls (12 hr light / 12 hr dark), and fed standard laboratory chow and water ad libitum.

The rats were divided into four equal groups. Group 1 did not receive melatonin or irradiation (control group) but received both 0.1 ml saline intraperitoneally (IP) and sham-irradiation. Group 2 received to total cranium 5 Gy of gamma irradiation as a single dose (IR group) plus 0.1 ml saline IP. Group 3 received irradiation to total cranium plus 5 mg/kg/day melatonin (IR+Mel group). Group 4 received only 5 mg/kg/day melatonin (Mel group) plus sham-irradiation.

The rats in the IR+Mel and Mel groups received 5mg/kg/day melatonin (Sigma Chemical Co, St. Louis USA) daily by intraperitoneal injection starting from first dose before 30 minutes irradiation and during 10 days after irradiation (total 10 days). Both the control group and the IR group were injected 0.1 ml saline daily by intraperitoneal injection starting from before irradiation and during 10 days after irradiation (total 10 days).

Prior to total cranium irradiation, the rats were anesthetized with 80 mg/kg ketamin HCl (Pfizer İlaç, İstanbul, Turkey) and placed on a Plexiglas tray in a prone position. While the rats in the control and Mel groups received sham-irradiation, the rats in the IR and the IR+Mel groups were irradiated using a cobalt-60 teletherapy unit (Picker, C-9, Maryland, NY, USA) from a source-to-surface distance of 80 cm, by $5 \times 5$ cm anterior fields with 5 Gy to the total cranium as a single fraction. To increase lens dose to maximum, a wax bolus material which has 0.5 cm thickness was placed on the rats eyes. The dose was calculated for the central axis at a depth of 0.5 cm. The dose rate was 0.59 Gy/min.

Determination of clinical cataract

In this study, the lens opacities classification system, version III (LOCS III) was used in the cataract classification. The lenses were graded by slit-lamp microscopy (Nikon, Zoom-Photo Slit Lamp, FS-3V, and Japan) as follows: The features of nuclear opacification and brunesence are graded according to 1 set of 6 photographs. The brightness of scatter from the nuclear region has been designated nuclear opalescence (NO) and the intensity of brunesence, nuclear color (NC). The amount of cortical cataract (C) is determined by comparing the estimated aggregate of cortical spoking to that seen in 5 separate photographs. Similarly, the estimated amount of posterior subcapsular cataract (P) is determined by comparing it to another 5 photographs depicting increasing amounts of posterior subcapsular cataract. At the beginning of the experiment, the lenses of rats in Experimental Animal Laboratory were examined by slit-lamp bio microscopy (Nikon, Zoom-Photo Slit Lamp, FS-3V, Japan), and forty rats, theirs lenses without cataract (NO, NOC, C, and P0), were randomly selected for this study.

Fractionation of lens samples

10 days after irradiation, the rats were anesthetized with ether at first. Then an intra-cardiac withdrawal of blood was performed. Following the withdrawal of blood, the rats were killed using a high dose of ether, and their eyes were enucleated and the lenses were dissected out immediately. Lenses were homogenized by an OMNI TH International, model TH 220 (Warrenton, VA 20187 USA) homogenizer in isotonic saline (1/20 weight/volume) on ice for 10 seconds in the first speed level. The homogenate was centrifuged at 10 000 g for 60 min at 4°C. The supernatant was stored at −80°C in aliquots for biochemical measurements. Activities of the antioxidant enzymes, SOD and GSH-Px and MDA level were determined from these supernatants spectrophotometrically.

Determination of MDA levels

Measurement of the MDA levels was carried out the method published by Ohkawa et al. In this method, samples less than 0.2 mL of 10% (w/v) tissue homogenate were added 0.2 mL of 8.1% sodium dodecyl sulfate (SDS), 1.5 mL of 20% acetic acid solution (whose pH was adjusted to
3.5 with NaOH), and 1.5 mL of 0.8% aqueous solution thiobarbituric acid (TBA). The mixture was made up to 4.0 mL with distilled water, and heated at 95°C for 60 minutes. After cooling, 1.0 mL of distilled water and 5.0 mL of the mixture of n-butanol and pyridine mixture (15:1, v/v) were added and the sample was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm was measured. Total thiobarbituric acid-reactive substances (TBARS) were expressed as MDA, using a molar extinction coefficient for MDA of 1.56 × 10^5 cm⁻¹ M⁻¹. MDA level was expressed as nmol/mg protein.

**Determination of SOD activity**

SOD activity was detected according to Sun and co-workers. In this method, xanthine-xanthine oxidase complex produces superoxide radicals, which react with nitrobluetetrazolium (NBT) to form the formazan compound. SOD activity is measured at 560 nm by detecting the inhibition of this reaction. By using a blank study in which all reagents except the supernatant sample was present and by determining the absorbance of sample and blank, the activity was calculated and given below. One SOD unit was defined as the enzyme amount causing 50% inhibition in the NBTH₂ reduction rate. SOD activity was also expressed as U/mg protein of lens sediment.

**Determination of GSH-Px activity**

GSH-Px activity was measured according to the Paglia and Valentina method. In this method, GSH-Px catalyses the oxidation of glutathione in the presence of hydrogen peroxide. Oxidized glutathione is converted into the reduced form in the presence of glutathione reductase and NADPH, while NADPH is oxidized to NADP. The reduction in the absorbance of NADPH at 340 nm is measured. By measuring the absorbance change per minute and by using the molar extinction coefficient of NADPH, GSH-Px activity of lens tissue was calculated. GSH-Px activities were expressed as milli-international unit (mU)/mg protein of lens sediment. The protein content was determined by using the Bradford method. Biochemical measurements were carried out at room temperature using a spectrophotometer (CECIL CE 3041, Cambridge, UK).

**Statistical Analyses**

The results were given as the median (minimum-maximum). In the study in which we planned to evaluate the grade of cataract and antioxidant enzymes levels in lens depending on irradiation in the rats; statistical analysis were made by using the SPSS packed program (Statistical Package for Social Science; Windows version 10.0) after the necessary data had been collected. Cataract development in the different treatment groups were compared with Fischer’s exact Chi-square test. Kruskal-Wallis non parametric analysis of variance/orthogonal Mann Whitney tests were used to examine for contrasts among the experiment modalities. When it was \( P < 0.05 \), it was accepted as statistically significant.

**RESULTS**

Lens grades by slit-lamp biomicroscopy are presented in Table 1. At the end of the experiment, while cataract development was detectable in 9 rats in the IR group, it was detectable only in 3 rats in the IR+Mel group, whereas none of the rats both in the control group and Mel group exhibited any biomicroscopic change in their lenses. Compared to in the IR group, significant reduction of cataract formation was observed in the IR+Mel group \( (p < 0.05) \). We observed a significant increase of cataract formation in the IR group, when compared to the control group \( (p < 0.05) \).

**Table 1. Cataract situation in the rat lenses examined by slit-lamp microscopy in the control, Mel, IR and IR+Mel groups.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Presence</th>
<th>Absent</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Melatonin</td>
<td>0</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Irradiated</td>
<td>9</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Irradiated plus melatonin</td>
<td>3</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>28</td>
<td>40</td>
</tr>
</tbody>
</table>

Fischer’s exact Chi-square test = 26.2 \( p < 0.001 \)

The level of MDA and the activity of SOD, GSH-Px in the rat lenses are all presented in Table 2. In the IR group, MDA level was higher than those of the other groups. There was a statistically significant difference between the IR group and the other groups \( (p < 0.05) \). There was no significant difference between the control group and the IR+Mel and Mel groups. In the control and the IR+Mel and Mel groups, the SOD activity was higher than that of the IR group and this level was higher in the IR+Mel and Mel groups than in the control group. There was statistical significance between the IR group and the other groups \( (p < 0.05) \) but there was no statistically significant difference found between the control group and the IR+Mel group \( (p > 0.05) \). There was also statistical significance between the control group and the Mel group \( (p < 0.05) \). In the control and the IR+Mel and Mel groups, GSH-Px activity was higher than that of the IR group \( (p < 0.05) \), and this level was higher in the IR+Mel and Mel groups than in the control group. There was a statistically significant difference between the IR group and the other groups \( (p < 0.05) \). There was also statistical significance between the control and the IR+Mel and the Mel groups \( (p < 0.05) \).
DISCUSSION

The goal of radiation treatment is to deliver completely measured doses of ionizing radiation to a defined tumor volume with the minimum accepted injurious effects of ionizing radiation to surrounding healthy tissue by eliminating tumor cells, giving a high quality of life and prolongation of survival at reasonable cost to cancer patients.\textsuperscript{24}) But, cataract is an unavoidable complication if radiotherapy includes the orbit in the treated volume, even with very low doses of radiation.\textsuperscript{25}) Ionizing radiation, such as X and gamma (\(\gamma\))-rays and ultraviolet lights, is known to be a cataractogenic factor for rat lenses.\textsuperscript{1,4,5,25}) Because damaging effects of ionizing radiation on living cells are predominantly due to ROS generated by the decomposition of water and/or Fenton reaction,\textsuperscript{1,5,26,27}) the theory of oxidative damage for cataract development is of interest.\textsuperscript{1,4,5}) Consequently, in a previous study, we determined that irradiation significantly increased both the MDA level and the activity of the GSH-Px, and significantly decreased the activity of the SOD in the rat lenses, indicating the generation of oxidative stress and an early protective response to oxidative damage.\textsuperscript{3}) Bardak et al.\textsuperscript{4,5}) found that one week after exposure, SOD and GSH-Px activities in the rat lenses were lower in the UVB group than in the controls, and the MDA level was higher than in the controls (\(P < 0.05\)), which served as an index of cellular damage by free radicals. They suggested that the depletion of important intracellular antioxidant stores by UV-radiation in the lenses of the animals might have been the main cause of lens opacification. We demonstrated that irradiation with 5 Gy to the total cranium as a single fraction formed grade 2 cataract formation at 10 days after post-irradiation in the rat lenses.\textsuperscript{1,4,5,25}) It was also reported that irradiation of 5 Gy in a single dose to the whole body significantly increased grade 3 cataract formation at 8 weeks after post-irradiation.\textsuperscript{28}) In the present study, the results of RT group were similar to the results of our previous study.

Melatonin scavenges OH\(^{•}\), nitric oxide (NO\(^{•}\)), O\(^{2−}\), and singlet oxygen (\(1\text{O}_2\)), a high energy form of O\(_2\) that exhibits high toxicity at the molecular level. Melatonin may decrease the quantity of O\(^{2−}\) in two ways, directly by stimulating SOD and indirectly when the melatonyl cation radical scavenges it. Melatonin stimulates the activity of GSH-Px, which transforms H\(_2\text{O}_2\) to O\(_2\).\textsuperscript{17}) The marked protective effects of melatonin against oxidative stress are aided by its ability to cross all biological membranes.\textsuperscript{18,29}) In the present study, the lenses of the rats injected with only melatonin (Mel group), the level of MDA as an index of lipid peroxidation was slightly lower than that of the control. But, this result was no statistically significant difference. This may indicate that the effects of melatonin on the biological membranes are limited under physiologic and normal condition. The activity of SOD and GSH-Px enzymes was significantly higher than that of the control and the other groups, indicating directly stimulant effects of the melatonin on these enzymes. Melatonin injection to rat did not cause cataract formation. Currently, there are increasing evidences, from experimental studies, suggesting that melatonin could be a beneficial agent in the protection against ionizing radiation-related

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA Median (min-max) nmol/mg protein</th>
<th>SOD Median (min-max) mU/mg protein</th>
<th>GSH-Px Median (min-max) mU/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17.90\textsuperscript{a} (10.80–25.60)</td>
<td>35.20\textsuperscript{b,c} (23.50–62.10)</td>
<td>273.10\textsuperscript{a,d} (142–565)</td>
</tr>
<tr>
<td>Melatonin</td>
<td>16.64\textsuperscript{a} (10.58–20.48)</td>
<td>47.61\textsuperscript{b,c,d} (40.61–72.55)</td>
<td>462.25\textsuperscript{b,c,d} (218–704)</td>
</tr>
<tr>
<td>Irradiated</td>
<td>26.00\textsuperscript{a,b,d} (16.90–30.20)</td>
<td>23.90\textsuperscript{b,d} (8.10–35.00)</td>
<td>164.43\textsuperscript{b,d} (105–243)</td>
</tr>
<tr>
<td>Irradiated plus melatonin</td>
<td>18.66\textsuperscript{a} (11.13–26.79)</td>
<td>38.35\textsuperscript{b,c} (24.64–58.37)</td>
<td>384.75\textsuperscript{a,b,c} (223–594)</td>
</tr>
</tbody>
</table>

MDA; malondialdehyde, SOD; superoxide dismutase, GSH-Px; glutathione peroxidase
\(\textsuperscript{a}P < 0.05\) compared to control group
\(\textsuperscript{b}p < 0.05\) compared to Mel group
\(\textsuperscript{c}P < 0.05\) compared to IR group
\(\textsuperscript{d}P < 0.05\) compared to IR+Mel group

Table 2. The level of MDA and the activity of SOD and GSH-Px in the rat lenses.
normal tissue injury, including lenses. Melatonin is a new class of radioprotectors against total body irradiation lethality. It was recently shown that whole-body irradiation caused multiple organ damage and melatonin, by its free radical scavenging and antioxidative properties, appeared to ameliorate irradiation-induced organ injury such as the liver, lung, colon, ileum and brain. In our study, irradiation significantly increased the activity of the SOD and the GSH-Px enzymes in the rat lenses. Melatonin supplementation with irradiation significantly increased the activity of SOD and GSH-Px enzymes and significantly decreased the MDA level. That is, total cranial irradiation of 5 Gy in single dose caused eye lens damage, and melatonin was able to protect against the damage produced by radiation with the up-regulation of antioxidant enzymes and by scavenging free radicals generated by ionizing radiation. Thus, our results suggest that supplementing cancer patients with adjuvant therapy of melatonin may improve patients suffering from toxic therapeutic regimens such as chemotherapy or radiotherapy or in combination with two and may provide an alleviation of the symptoms due to radiation-induced organ injuries.

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