Radio-protective effects of melatonin against irradiation-induced oxidative damage in rat peripheral blood

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Abstract During radiotherapy, ionizing irradiation interacts with biological systems to produce free radicals, which attacks various cellular components. The hematopoietic system is well-known to be radiosensitive and its damage may be life-threatening. Melatonin synergistically acts as an immunostimulator and antioxidant. In this study we used a total of 120 rats with 20 rats in each group. Group 1 did not receive melatonin or irradiation (Control group), Group 2 received only 10 mg/kg melatonin (Mel group), Group 3 exposed to dose of 2 Gy irradiation (2 Gy Rad group), Group 4 exposed to 8 Gy irradiation (8 Gy Rad group), Group 5 received 2 Gy irradiation plus 10 mg/kg melatonin (Mel+2 Gy Rad group) and Group 6 received 8 Gy irradiation plus 10 mg/kg melatonin (Mel+8 Gy Rad group). Following exposure to radiation, five rats from each group were sacrificed at 4, 24, 48 and 72 h. Exposure to different doses of irradiation resulted in a dose-dependent decline in the antioxidant enzymes activity and lymphocyte count (LC) and an increase in the nitric oxide (NO) levels of the serum. Pre-treatment with melatonin (10 mg/kg) ameliorates harmful effects of 2 and 8 Gy irradiation by increasing lymphocyte count(LC) as well as antioxidant enzymes activity and decreasing NO levels at all time-points. In conclusion 10 mg/kg melatonin is likely to be a threshold concentration for significant protection against irradiation-induced oxidative damage in rat peripheral blood.
Introduction

Recently application of radiation science in different settings (e.g., radiotherapy, biomedical research, military and space research) is increased and therefore protecting humans against the harmful effects of radiation is a major challenge that needs an urgent solution. During radiotherapy, ionizing radiation interacts with biological systems to produce free radicals or reactive oxygen species (ROS), which attack various cellular components including DNA, proteins and membrane lipids, leading to serious cellular damage [1].

To control the flux of ROS, aerobic cells have developed their own defense system, the antioxidant system, which includes enzymatic and non-enzymatic components [2]. The antioxidant system consists of low-molecular-weight antioxidant molecules, such as glutathione (GSH), melatonin and various antioxidant enzymes [2]. For instance the antioxidant enzymes, superoxide dismutase (SOD), the first line of defense against oxygen-derived free radicals, catalyses the dismutation of the superoxide anion \( \text{O}_2^- \) into \( \text{H}_2\text{O}_2 \). Hydrogen peroxide can be transformed into \( \text{H}_2\text{O} \) and \( \text{O}_2 \) by catalase when it is present in peroxisomes of eukaryotic cells. Glutathione peroxidase (GSH-Px) is a selenoprotein, which reduces lipidic or nonlipidic hydroperoxides as well as \( \text{H}_2\text{O}_2 \) while oxidizing GSH [3,4].

There is increasing evidence that nitric oxide (NO), as well as its derivatives, may play a role in multistage carcinogenesis [5]. Nitric oxide, together with reactive oxygen species (ROS), is known to induce cytotoxicity and cytostasis. Various studies using NO and \( \text{H}_2\text{O}_2 \) induced oxidative damage have shown to induce similar cytotoxicity [6]. NO reacts rapidly with the superoxide anion (\( \text{O}_2^- \)) to form peroxynitrite (ONOO\(^-\)), which in itself is cytotoxic and readily decomposes into the highly reactive and toxic hydroxyl radical (\( \cdot\text{OH} \)) and nitrogen dioxide (\( \text{NO}_2 \)) [5].

The hematopoietic system is well-known to be radiosensitive and its damage may be life-threatening [2]. Hence, agents which protect the hematopoietic system and lymphoid cells from radiation-induced damage need to be identified. Moreover, it has been observed that the protection of normal tissues may provide an increase in tumor control by allowing for an increase in the radiation dose [7,8]. Various chemical compounds such as amifostine and other sulfhydryl compounds have been investigated as potential radioprotective agents [9]. However, the inherent toxicity of these agents at the radio-protective doses warranted further search for safer and more effective radioprotectors [9,10].

Melatonin synergistically acts as an immunostimulator [11,12] and antioxidant [13–17]. Moreover, due to small size and high lipophilicity, melatonin crosses biological membranes and reaches to all compartments of the cell [18].

Melatonin has been shown to be a direct free radical scavenger and indirect antioxidant via its stimulatory actions on antioxidant enzymes activity [13,16,19] and inhibitory actions on pro-oxidative enzymes activity [20]. Melatonin has been reported to inhibit the activity of nitric oxide synthase (NOS) [20–22], the enzyme catalyzing the formation of NO [20]. Thus, melatonin, by inhibiting NOS activity, decreases the formation of NO and the product of its interaction with \( \text{O}_2^- \), ONOO\(^-\) [20].

In this study, we investigated the possible radio-protective effects of pre-treatment with melatonin against whole-body-irradiation-induced oxidative damage on peripheral blood of rats at different time-points after exposure.

Material and method

The experimental protocol was in accordance with the guidelines for care and use of laboratory animals as adopted by the Ethics Committee of the School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.

Animal care and maintenance

Eight - to ten-week-old male Wistar rats, each weighing 180–220 g, were obtained from Department of Pharmacology Experimental Animal Laboratory, School of Medicine, Tehran University of Medical Sciences. They were housed in animal cages, with room temperature maintained at 20–22°C, relative humidity of 50–70% and an airflow rate of 15 exchange/h. Also, a time-controlled system provided 08:00–20:00 h light and 20:00–08:00 h dark cycles. All rats were given standard rodent chow diet and water from sanitized bottle fitted with stopper and sipper tubes.

Experimental design

After acclimatization for a week to laboratory conditions, the rats were divided into six different groups. Half an hour before the start of the experiment, all rats were transferred to a laboratory near the Cobalt 60-gamma irradiator (Theratron 780, Atomic energy of Canada limited, Canada) facility. In this study we used a total of 120 rats with 20 rats in each group. Group 1 did not receive melatonin or irradiation (Control group) but received both 5% absolute ethanol in 0.5 ml phosphate-buffered saline (PBS), as a vehicle, intraperitoneally (IP) and sham-irradiation. Group 2 received only 10 mg/kg body weight melatonin (Mel group) plus sham-irradiation. Group 3 exposed to dose of 2 Gy whole-body gamma irradiation (2 Gy Rad group) plus 5% absolute ethanol in 0.5 ml phosphate-buffered saline IP. Group 4 exposed to dose of 8 Gy whole-body gamma irradiation (8 Gy Rad group) plus 5% absolute ethanol in 0.5 ml phosphate-buffered saline IP. Group 5 received 2 Gy whole-body gamma irradiation plus 10 mg/kg melatonin (Mel + 2 Gy Rad group). Group 6 received 8 Gy whole-body gamma irradiation plus 10 mg/kg melatonin (Mel + 8 Gy Rad group). Rats in groups 2, 5 and 6 were given an intraperitoneal injection of freshly prepared melatonin (Sigma–Aldrich Co., St. Louis, MO, USA) in 0.5 ml of 5% absolute ethanol solution. Melatonin was first dissolved in lower dose of 2 Gy gamma irradiation compared to higher dose of 8 Gy. Therefore, it seems that radio-protective effects of melatonin are dose-dependent.
a small amount of absolute ethanol (25 μL) and then diluted with phosphate-buffered saline (PBS) in final ethanol concentration 5%. Half an hour after the injections, all of the rats were anesthetized with an intraperitoneal injection of ketamin (60 mg/kg) and xylazin (20 mg/kg), and then the rats in groups 3, 4, 5 and 6 were exposed to a whole-body gamma irradiation doses of 2 and 8 Gy at a dose rate of 101 cGy/min with a source surface distance (SSD) of 80 cm and fixed field size of 10 × 10 cm² at room temperature (22 ± 2 °C).

Following exposure to gamma irradiation, five rats from each group were sacrificed at 4, 24, 48 and 72 h. Blood was collected from heart puncture under ether anesthesia in heparinized tube for lymphocyte collection and non-heparinized tube for serum collection. Each blood sample was divided into two parts. One part was used for lymphocyte count and another part was used for measurement of antioxidant enzymes activities and nitric oxide levels in serum. Serums were frozen at −20 °C for the following measurements.

**Lymphocyte count (LC)**

Lymphocytes were isolated from each blood sample using Ficoll–Histopaque density gradients (Sigma, St. Louis, MO, USA) with modification. Blood was diluted 1:3 with phosphate-buffered saline (PBS) and layered on to the Histopaque in the ratio of 2:1 (blood + PBS: Histopaque). The blood was centrifuged at 400 × g for 20 min at room temperature. The lymphocytes layer was removed and then washed twice in PBS at 250 × g for 10 min each. Liquid layer was removed and then added 1 ml of PBS to sediment layer (lymphocytes layer) as a final sample. A thin layer of final sample was prepared on a glass slide and number of lymphocytes was counted by microscope (Olympus Optical Co. Ltd, Japan). LC was also expressed as 10⁶ cells/mL.

**Biochemical parameters**

All of the parameters assessments were operated according to instructions of BioVision assay kits (980 Linda Vista Avenue, Mountain View, CA 94043 USA) and determined by a colorimetric method with ILISA Microplate Reader (Bio Tek Instruments, Inc, USA).

Superoxide dismutase (SOD) assay kit, briefly, utilizes WST-1 that produces a water-soluble formazan dye upon reduction with superoxide anion. The rate of the reduction with a superoxide anion is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. Therefore, the activity of SOD can be determined by absorbance at 450 nm using a microplate reader. The SOD activity was also expressed as percent of inhibition rate (inhibition rate %).

Glutathione peroxidase (GSH-Px) Assay Kit, briefly, measures glutathione peroxidase (GSH-Px) activity through a coupled reaction with glutathione reductase (GR). In the assay, GSH-Px reduces Cumene Hydroperoxide, and oxidizes GSH to GSSG. The generated GSSG is reduced to GSH with consumption of NADPH by GR. The decrease of NADPH is proportionally to GSH-Px activity in the reactions. The decrease of NADPH can be measured by absorbance at 340 nm. The GSH-Px activities were expressed as mU/mL (One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 μmol of NADPH to NADP⁺ under the assay kit condition per minute at 25 °C). Catalase (CAT), briefly, can be determined by this manner, catalase first reacts with H₂O₂ to produce water and oxygen, the unconverted H₂O₂ reacts with OxiRed™ probe to produce a product, which can be measured at 570 nm. Catalase activity is reversely proportional to the signal. The CAT activity was also expressed as mU/mL. (One unit of catalase is the amount of catalase decomposes 1.0 μmol of H₂O₂ per min at pH 4.5 at 25 °C). Nitric Oxide (NO) Colorimetric Assay Kit provides a measure of total nitrate or nitrite in two-step process. The first step converts nitrate to nitrite utilizing nitrate reductase. The second step uses Griess Reagents to convert nitrite to a deep purple azo compound. The amount of the azo chromophore accurately reflects nitric oxide amount in samples. Nitric oxide level can be determined as a function of nitrate concentration by absorbance at 540 nm. The NO level was expressed as nitrate nmol/mL.

**Statistical analysis**

Each data point represents mean ± standard error on the mean (SEM) of at least five animals per group. A one-way analysis of variance (ANOVA) was performed to compare different groups, followed by Tukey’s multiple comparison tests. *P* < 0.05 was considered to represent a statistically significant difference.

**Results**

The lymphocyte count, antioxidant enzymes activities and nitric oxide levels of six groups at all time-points are given in Figs. 1—5.

**Lymphocyte count (LC)**

As can be seen in Fig. 1, LC in 2 and 8 Gy Rad groups was significantly decreased when compared with control group at all time-points of post-irradiation (*p* < 0.05). The values for 8 Gy Rad group were less than 2 Gy Rad group at all time-points and was statistically significant only at 4 h post-irradiation. Pre-treatment with melatonin (10 mg/kg) ameliorates the harmful effects of irradiation. The LC in Mel + 2 or 8 Gy Rad group was higher compared to 2 or 8 Gy Rad exposed group. The values were statistically significant at all time-points of 2 Gy post-irradiation and only at 4 h of 8 Gy post-irradiation.

**Superoxide dismutase (SOD) activity**

As shown in Fig. 2, SOD activity in the irradiated groups (2 and 8 Gy Rad) decreased significantly when compared with control group at all time-points of post-irradiation (*p* < 0.05) except at 24 h exposure to 2 Gy. SOD activity of 8 Gy Rad group was significantly lower than 2 Gy Rad group at all time-points (*p* < 0.05). Pre-treatment with melatonin (10 mg/kg) increases the SOD activity of 2 and 8 Gy irradiation at all time-points. The values were, however, statistically significant at the sampling times 4 and 24 h of both 2 and 8 Gy.
Glutathione peroxidase (GSH-Px) activity

As shown in Fig. 3, GSH-Px activity in the irradiated groups (2 and 8 Gy Rad) decreased significantly when compared with control group at all time-points of post-irradiation ($p < 0.05$). GSH-Px activity for 8 Gy Rad group was significantly lower than 2 Gy Rad group at all time-points ($p < 0.05$) except at 48 h post-irradiation. Results obtained from Fig. 3 indicated that pre-treatment with melatonin (10 mg/kg) ameliorates the deleterious effects of 2 and 8 Gy irradiation by increasing GSH-Px activity at all time-points. However, this increase was not significant at any of the time-points of 8 Gy post-irradiation. Pre-treatment with melatonin (10 mg/kg) significantly increased the GSH-Px activity when compared with 2 Gy Rad group at all time-points other than 24 h post-irradiation ($p < 0.05$).

Catalase (CAT) activity

As shown in Fig. 4, CAT activity of irradiated groups (2 and 8 Gy Rad) decreased significantly when compared with control group at all time-points of post-irradiation...
CAT activity for 8 Gy Rad group was significantly lower than 2 Gy Rad group at all time-points. Pre-treatment with melatonin (10 mg/kg) ameliorates the injurious effects of 2 and 8 Gy irradiation by increasing the CAT activity at all time-points. However, majority of these increases were not statistically significant except for 8 Gy at 24 h post-irradiation ($p < 0.05$).

Nitric oxide (NO) level

As shown in Fig. 5, pre-treatment with melatonin (10 mg/kg) ameliorates the deleterious effects of 2 and 8 Gy irradiation by significant decrease in NO levels at all time-points ($p < 0.05$). Moreover, NO levels of irradiated groups (2 and 8 Gy Rad) were significantly high ($p < 0.05$) when compared with control group at all time-points of post-irradiation. A maximum value was observed at 4 h after exposure to 8 Gy irradiation. NO levels for 8 Gy Rad group were significantly higher than 2 Gy Rad group at all time points ($p < 0.05$).

Discussion

Several studies demonstrated that melatonin by antioxidant properties, appeared to ameliorate irradiation-induced injury in various organs including brain [23],

![Figure 2](image-url)
spinal cord [24–26], lens [27], liver [28–30], spleen [2,31,32], lung, colon and ileum [29].

In our present study, whole-body irradiation of rats to different doses of gamma radiation (2 and 8 Gy) resulted in a dose-dependent decrease in the SOD, GSH-Px, CAT activity as well as in LC and increase in the NO levels of the serum. The decrease in the activities of SOD, GSH-Px and CAT may be due to their utilization by the increased ROS production, and decrease in LC indicates the suppression in immune status of animals. Moreover, it seems that irradiation stimulates the activity of nitric oxide synthase (NOS), and increase in the levels of NO may be due to increase in the activity of NOS.

Results obtained from our study indicated that pre-treatment with melatonin (10 mg/kg) ameliorates the deleterious effects of 2 and 8 Gy irradiation by increasing the LC, the antioxidant enzymes activity and decreasing NO levels at all time-points. Although at most of the time-points, except for NO levels, these ameliorative effects of melatonin were not statistically significant for 8 Gy irradiation. Therefore, melatonin suppressed the reduced activities of antioxidant enzymes (SOD, GSH-Px and CAT), and stimulated immunity of rats by blocking the radiation-induced suppression on LC. Furthermore, it seems that, by inhibiting NOS activity, melatonin decreases the formation of NO.

Figure 3  Effect of melatonin pre-treatment (10 mg/kg) on GSH-Px activity of serum at 4, 24, 48 and 72 h after exposure to (a) 2 Gy and (b) 8 Gy irradiation. Vertical bars represent mean ± SEM, n = 5 for each group. Con, Control; Mel, Melatonin only; 2 Gy Rad, 2 Gy Irradiation only; Mel+2 Gy Rad, Melatonin treatment and 2 Gy irradiation; 8 Gy Rad, 8 Gy Irradiation only; Mel+8 Gy Rad, Melatonin treatment and 8 Gy irradiation. †p < 0.05 when compared with their respective control groups, *p < 0.05 when compared with their respective 2 Gy Rad groups and **p < 0.05 when compared with their respective 8 Gy Rad groups.
On the other hand, melatonin treatment maintained LC, antioxidant enzymes activity and NO levels close to control groups and showed insignificant changes compared to control groups except at 4 h for lymphocyte count and NO level. Thus, administration of melatonin (10 mg/kg) did not induce serious side effects and acute toxicity.

Koc et al. [33] investigated the antioxidant role of melatonin (at 5 and 10 mg/kg) in the liver tissue against total-body gamma irradiation-induced oxidative damage with a single dose of 6.0 Gy. The results demonstrated that in irradiated rats, that were pretreated with melatonin (5 or 10 mg/kg), malondialdehyde (MDA) levels, as an end product of lipid peroxidation in the liver tissue, were significantly lowered, whereas the SOD and GSH-Px activities were significantly increased. They concluded that pre-treatment with melatonin may prevent irradiation-induced liver damage [33].

After exposure to 6 Gy Whole-body irradiation, liver malondialdehyde (MDA) and nitric oxide (NO) levels were measured by Taysi et al. [30]. Gamma irradiation caused a significant increase in liver MDA and NO levels. Hepatic MDA and NO levels in irradiated rats that were pretreated with melatonin (5 or 10 mg/kg) were significantly decreased [30]. Furthermore, the antioxidant property of melatonin (5 mg/kg, administrated daily for 10 days before irradiation) against a single dose of 5 Gy total-cranium irradiation was demonstrated by Koc et al. [33].

Figure 4  Effect of melatonin pre-treatment (10 mg/kg) on CAT activity of serum at 4, 24, 48 and 72 h after exposure to (a) 2 Gy and (b) 8 Gy irradiation. Vertical bars represent mean + SEM, n = 5 for each group. Con, Control; Mel, Melatonin only; 2 Gy Rad, 2 Gy Irradiation only; Mel+2 Gy Rad, Melatonin treatment and 2 Gy irradiation; 8 Gy Rad, 8 Gy Irradiation only; Mel+8 Gy Rad, Melatonin treatment and 8 Gy irradiation. *p < 0.05 when compared with their respective control groups, *p < 0.05 when compared with their respective 2 Gy Rad groups and **p < 0.05 when compared with their respective 8 Gy Rad groups.
irradiation-induced cataract in the lens of rats was investigated [27]. Irradiation significantly increased the MDA level and also significantly decreased SOD and GSH-Px activity, emphasizing the generation of increased oxidative stress. Melatonin supplementation with irradiation significantly increased the activity of SOD and GSH-Px enzymes and significantly decreased the MDA level [27]. El-Missiry et al. [28] showed that treatment with 10 mg/kg melatonin for 4 days (daily) before acute irradiation (2 and 4 Gy) significantly reduced radiation-induced increases in MDA and protein carbonyl levels (the oxidative stress markers) in the liver and significantly maintained hepatic glutathione (GSH) content, glutathione-S-transferase (GST), and catalase (CAT) activities close to the control group values.

Sharma et al. showed that, due to its antioxidant properties, melatonin increased the immunity in squirrels, by protecting their hematopoietic system and lymphoid organs against 2.06 Gy X-ray-induced cellular toxicity [31]. In their study, Total leukocyte and lymphocyte counts (TLC and LC) in the peripheral blood and lipid peroxidation (LPO) status, superoxide dismutase (SOD) activities and total antioxidant status (TAS) were measured in the spleens of squirrels. pretreatment with melatonin prior to the irradiation significantly increased LC, TLC, SOD activity and TAS status compared to irradiation exposed groups whereas LPO status

Figure 5 Effect of melatonin pre-treatment (10 mg/kg) on NO levels of serum at 4, 24, 48 and 72 h after exposure to (a) 2 Gy and (b) 8 Gy irradiation. Vertical bars represent mean + SEM, n = 5 for each group. Con, Control; Mel, Melatonin only; 2 Gy Rad, 2 Gy Irradiation only; Mel+2 Gy Rad, Melatonin treatment and 2 Gy irradiation; 8 Gy Rad, 8 Gy Irradiation only; Mel+8 Gy Rad, Melatonin treatment and 8 Gy irradiation. *p < 0.05 when compared with their respective control groups, **p < 0.05 when compared with their respective 2 Gy Rad groups and ***p < 0.05 when compared with their respective 8 Gy Rad groups.
was decreased [31]. In another study, a radio-protective effect of melatonin against 5 Gy gamma irradiation during the reproductively active and inactive phases (RAP and RIP) of Indian palm squirrels was evaluated. Results showed that melatonin pre-treatment significantly increased the LC and increased SOD activity in the spleen of squirrels compared with irradiation groups[32].

The results of treatment with melatonin followed by radiation exposed groups were similar to the results of the reported studies, where pre-treatment with melatonin increased SOD, GSH-Px and CAT activity and decreased the levels of NO [27,28,30–33]. Our results of LC are in agreement with that of Sharma et al. [2,31,32] studies when reported that pre-treatment with melatonin increased lymphocyte count in irradiated groups. Thus our results support the findings of previously published literature. Melatonin pre-treatment increased LC, the activity of the antioxidant enzymes and decreased the levels of nitric oxide. Furthermore, to our knowledge, there have been no published studies investigating possible radio-protective effects of melatonin on peripheral blood (serum) by measuring activities of these antioxidant enzymes (SOD, GSH-Px and CAT) and levels of nitric oxide (NO) at different time-points after exposure to different doses of whole-body gamma irradiation.

Despite the lack of clinical and experimental studies the result of the present study, our previous data [24–26,34], and findings of other investigators, suggest that administration of this agent may enable the use of higher doses of irradiation during radiotherapy and may be beneficial in alleviating the complications of cancer treatment.

Conclusion

Based on our results, ionizing radiation causes dose-dependent oxidative damage on the hematopoietic system and melatonin, due to its immune stimulatory and antioxidant properties, ameliorates radiation-induced injury to this system. In conclusion 10 mg/kg melatonin is likely to be an adequate concentration for offering significant protection against lower dose of gamma irradiation (2 Gy) than higher dose of 8 Gy. Long-term administration of this drug may produce more protection against higher doses of irradiation, because lower dose of melatonin might not be enough to scavenge all the free radicals generated by higher dose of irradiation and stimulate cellular antioxidant defenses. Therefore, it seems that radio-protective effects of melatonin are dose-dependent. However, further experiments and clinical trials on this subject are still necessary to validate it.

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References


