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Melatonin may play a role in modulation of bax and bcl-2 expression levels to protect rat peripheral blood lymphocytes from gamma irradiation-induced apoptosis

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ABSTRACT

The close relationship between free radicals effects and apoptosis process has been proved. Melatonin has been reported as a direct free radical scavenger. We investigated the capability of melatonin in the modification of radiation-induced apoptosis and apoptosis-associated upstream regulators expression in rat peripheral blood lymphocytes. Rats were irradiated with a single whole body Cobalt 60-gamma radiation dose of 8 Gy at a dose rate of 101 cGy/min with or without melatonin pretreatments at different concentrations of 10 and 100 mg/kg body weight. The rats were divided into eight groups of control, irradiation-only, vehicle-only, vehicle plus irradiation, 10 mg/kg melatonin alone, 10 mg/kg melatonin plus irradiation, 100 mg/kg melatonin alone and 100 mg/kg melatonin plus irradiation. Rats were given an intraperitoneal (IP) injection of melatonin or the same volume of vehicle alone 1 h prior to irradiation.

Blood samples were taken 4, 24, 48 and 72 h after irradiation for evaluation of flow cytometric analysis of apoptotic lymphocytes using Annexin V/PI assay and measurement of bax and bcl-2 expression using quantitative real-time PCR (RT^2qPCR). Irradiation-only and vehicle plus irradiation showed an increase in the percentage of apoptotic lymphocytes significantly different from control group ($P < 0.01$), while melatonin pretreatments in a dose-dependent manner reduced it as compared with the irradiation-only and vehicle plus irradiation groups ($P < 0.01$) in all time points. This reduced apoptosis by melatonin was related to the downregulation of bax, upregulation of bcl-2, and therefore reduction of bax/bcl-2 ratio. Our results suggest that melatonin in these doses may provide modulation of bax and bcl-2 expression as well as bax/bcl-2 ratio to protect rat peripheral blood lymphocytes from gamma irradiation-induced apoptosis.

1. Introduction

Ionizing radiation is known to induce oxidative stress via the generation of reactive oxygen species (ROS) and free radicals in irradiated tissue and cells [1,2]. These free radicals react with DNA, proteins and lipids in nucleus and biological membranes [3–5]. Significant changes in structure and function of DNA (DNA double strand breaks) and membranes (more rigidity) result in cell death via apoptosis [6,7]. The p53 gene appears to be critical in initiating apoptosis particularly in apoptotic process related to DNA damaging stimulus like radiation [8–12]. The p53 activation is caused by upregulation of bax and its translocation from cytoplasm to mitochondria. On the other hand, anti-apoptotic members of the Bcl-2 family such as bcl-2 counteract the actions of bax or other pro-apoptotic proteins at the level of the mitochondria. The change in bax/bcl-2 ratio mediated the mitochondrial damage by breakdown of the mitochondrial transmembrane potential and release of cytochrome c into the cytoplasm [8–12] which caused a caspase cascade and finally led to the generation of an apoptotic phenotype [13]. Anyway, the relative expression of pro- and anti-apoptotic Bcl-2 family members is the key decision point regarding cell death induction [12,14]. Takahashi et al. [15] observed the accumulation of p53 and Bax, and the induction of apoptosis in mouse splenic lymphocytes 12 h after whole-body irradiation with X-rays

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at 3.0 Gy. In other study, Alvarez et al. [16] found that the expression of genes involved in the two apoptotic pathways was reached to maximum at 3 h post-irradiation in thymic and splenic lymphocytes of gamma irradiated mouse with a maximum in the number of apoptotic cells at 6 h. It has also been shown that lymphocytes readily undergo apoptosis in patient treatment with ionizing radiation [17]. Cui et al. [18] showed that 4 h after whole body 2–8 Gy of gamma irradiation, the apoptotic lymphocytes increased rapidly and the abnormal expression of bax and bcl-2 in irradiated lymphocytes was closely related to apoptosis in mouse peripheral blood lymphocytes.

Since radiation-induced cellular injury is attributed primarily to the harmful effects of free radicals, molecules with direct free radical scavenging properties are especially promising as radioprotectors [19,20]. Melatonin (N-acetyl-5-methoxytryptamine), the main secretory product of the pineal gland, has an important role as a direct and indirect free radical scavenger by neutralizing toxic reactive oxygen species (ROS) and reactive nitrogen species [21–23]. These mechanisms require presence of melatonin at the time of radiation in cells and tissues [19,20]. The widespread distribution of melatonin in subcellular compartments such as cytosol, nucleus, mitochondria, and cellular membrane has allowed it to effectively protect various normal cells from oxidative damages induced by ionizing radiation [24,25]. Koc et al. [26] showed that melatonin administration prior to irradiation prevented gamma radiation-induced damage on rat peripheral blood cells in vivo. Vijayalaxmi et al. proved that melatonin protects or reduces gamma radiation-induced chromosome damage, micronuclei and primary DNA damage in human peripheral blood lymphocytes in their in vitro and in vivo/in vitro investigations [27–29]. However, these findings indirectly suggest that melatonin may have protective effects against ionizing radiation-induced apoptosis in peripheral blood lymphocytes.

The aim of the present study is to investigate the modulating effect of melatonin on gamma radiation-induced apoptosis and the differences in expression of bax and bcl-2, the key upstream regulators of apoptosis, in rat peripheral blood lymphocytes.

2. Materials and methods

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.

2.1. Rats and maintenance

Eight- to ten-week-old male Wistar rats, each weighing 180–200 g, were obtained from Tehran University Animal Facility, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran. They were housed in Animal facility, 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.

2.2. Experimental design and irradiation

After 2 week acclimatization period, a randomized block design based on the animal body weights was used to divide the rats into eight different groups. The experiment was executed using a total of 160 rats with 20 rats in each of the following eight groups:

Group 1: Control.
Group 2: 8 Gy whole body gamma radiation.
Group 3: Vehicle (5% absolute ethanol in phosphate-buffered saline).
Group 4: Vehicle + 8 Gy whole body gamma radiation.
Group 5: Melatonin (10 mg/kg body weight).
Group 6: Melatonin (10 mg/kg body weight) + 8 Gy whole body gamma radiation.
Group 7: Melatonin (100 mg/kg body weight).
Group 8: Melatonin (100 mg/kg body weight) + 8 Gy whole body gamma radiation.

One hour before the start of experiment, all rats were transferred to a laboratory near the Cobalt 60-gamma irradiator (Theratron 780, Atomic Energy of Canada limited, Canada) facility. Control rats in group 1 did not received melatonin or irradiation but received both an intraperitoneal (IP) injection of 500 μl of phosphate-buffered saline (PBS) and sham-irradiation. Rats in group 2 were untreated and irradiated and received the same volume of PBS 1 h prior to irradiation. Rats in groups 3–8 were given an IP injection of freshly prepared melatonin (Sigma–Aldrich Co., St. Louis, MO, USA) in 500 μl of 5% absolute ethanol solution or the same volume of vehicle alone [30]. Melatonin was first dissolved in a small amount of absolute ethanol (25 μl) and then diluted with phosphate-buffered saline (475 μl) in final ethanol concentration 5%. One hour after the injections, all rats were anesthetized with an IP injection of ketamin (60 mg/kg) and xylazin (20 mg/kg), and then the rats in groups 2, 4, 6 and 8 were exposed to a whole-body gamma radiation dose of 8 Gy at a dose rate of 101 cGy/min with a source surface distance (SSD) of 80 cm and fixed field size of 35 cm × 35 cm at room temperature (22 ± 2 °C). The selection of 1 h interval between melatonin injection and exposure to gamma radiation was largely based on previous studies [30,31,38]. Also, the melatonin concentrations and the dose of gamma radiation selected were based on the experience from the studies performed by other investigators [30–32].

Under ketamin (50 mg/kg) and xylazin (10 mg/kg) anesthesia, three blood samples (each blood sample contained 2 ml of peripheral blood from each animal) were taken on EDTA sterile tubes from each group at each of the collection times of 4, 24, 48 and 72 h post irradiation. Each blood sample was divided into two parts. One part was used for evaluation of flow cytometric analysis of apoptotic lymphocytes using Annexin V/propidium iodide (PI) double staining and another part was used for measurement of bax and bcl-2 expression levels using quantitative real-time reverse transcriptase-polymerase chain reaction (RT^2qPCR).

2.3. Flow cytometric analysis of apoptotic lymphocytes

Lymphocytes were isolated from blood sample using Ficoll-Histopaque density gradients (Sigma, St. Louis, MO, USA) as described previously [33] with slight modification. Blood was diluted 1:3 with phosphate-buffered saline (PBS) and carefully layered onto the Histopaque in the ratio of 2:1 (blood + PBS:Histopaque). The blood was centrifuged at 400 × *g* for 25 min at room temperature. The lymphocyte layer was removed and washed three times in PBS at 250 × *g* for 10 min each, and then washed with RPMI 1640 medium. Typically, each lymphocyte sample consisted of an initial density of 1×10^6 cells/ml. Apoptosis as well as necrosis was evaluated using the Annexin-V-FLUOS Staining Kit (Roche Diagnostics GmbH, Penzberg, Germany) according to the instructions of the manufacturer. Briefly, Annexin-V-FLUOS labeling solution was prepared by prediluting 2 μl Annexin-V-Fluos labeling reagent in 100 μl incubation buffer and by adding 2 μl propidium iodide (PI) solution for each sample. Lymphocytes were then resuspended in 100 μl of Annexin-V-FLUOS labeling solution and incubated at room temperature in the dark for 15 min. Also, in each time point, a negative control lymphocyte sample was obtained without the staining procedure for identification of quadrant. Data from each lymphocyte sample were acquired immediately after the staining procedure. Lymphocyte samples were analyzed for the presence of apoptotic and necrotic cells by flow cytometry on a FACS Calibur flow cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with Cell Quest software using 488 nm excitation and a 515 nm bandpass filter (FL1) for Annexin-V-Fluos detection and a filter >600 nm (FL3) for PI detection. For each group at the indicated intervals following the treatment with gamma irradiation of 8 Gy at least three independent lymphocyte samples were analyzed. In each sample, a minimum of 10,000 events were counted and analyzed.

2.4. Quantitative real-time RT-PCR

Quantitative real-time PCR (RT^2qPCR) was used to measure the expression of pro-apoptotic bax and anti-apoptotic bcl-2 genes. In each sample, lymphocytes were isolated from 500 μl EDTA-preserved fresh whole blood using Ficoll-Histopaque as described earlier. RNA, then, was prepared with the High Pure RNA Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. RNA purity was quantified by spectrophotometry at 260/280 nm ratio and the integrity was confirmed by electrophoresis on a denaturing agarose gel. For each RNA sample, absence of contaminating DNA was examined by a PCR without preceding RT-reaction and no amplification product was observed. The cDNA was reverse transcribed from 1 μg total RNA with Expand Reverse Transcriptase (Roche Diagnostics GmbH, Mannheim, Germany) using random hexamer primers in a total volume of 20 μl. The mixture was incubated to synthesize cDNA for 10 min at 65 °C, to elongate cDNA for 10 min at 30 °C followed by for 45 min at 42 °C and to inactivate the enzyme for 2 min at 95 °C as recommended by the manufacturer. All rat primers were designed using ABI Primer Express software. The sequences of forward and reverse primers were as follows: Bax (NM_017059), forward primer: 5′-TGT TAC AGG GTT TCA TCC AG-3′, reverse primer, 5′-ATC CTC TGC AGC TCC ATA TT-3′, Bcl-2 (NM_016993), forward primer: 5′-GAT TGT GGC CTT CTT TGA GT-3′, reverse primer: 5′-ATA GTT CCA CAA AGG CAT CC-3′ and GAPDH forward primer: 5′-TTC ACC ACC ATG GAG AAG GC-3′, reverse primer: 5′-GGC ATG GAC TGT GGT CAT GA-3′. The level of expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene served as an internal control.

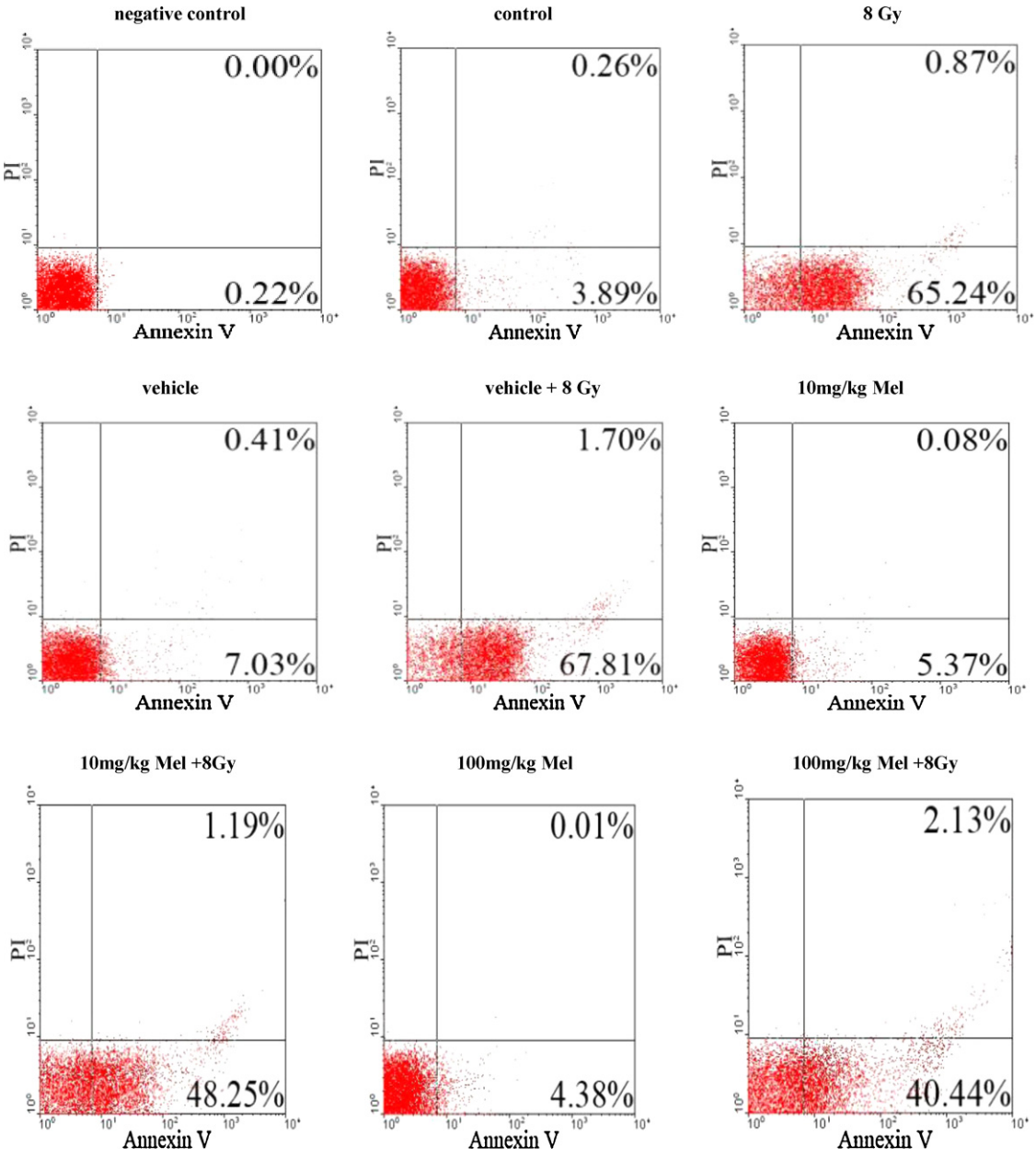

Fig. 1. Effect of melatonin on radiation-induced apoptosis in rats peripheral blood lymphocytes. Rats were exposed to a single whole body γ-ray dose of 8 Gy with or without melatonin (Mel) pretreatments (10 and 100 mg/kg IP 1 h before irradiation). Apoptotic and necrotic lymphocytes were analyzed by flow cytometric assay 4 h post irradiation. Representative dot plots of one set of three independent experiments of Annexin V and PI staining. Apoptotic lymphocytes (Annexin V^+ and PI^-) were displayed in the lower right quadrant and necrotic lymphocytes (Annexin V^+ and PI^+) were shown in the upper right quadrant.

Real-time quantitative RT-PCR amplifications were performed using Titan HotTaq EvaGreen® qPCR Mix (BioAtlas, Riia, Estonia) on the Bio-Rad real-time PCR detection system (Hercules, CA, USA). The real-time PCR mixture consisted of 2 μl cDNA and 300 nM each of forward and reverse primers in a total volume of 20 μl. The amplification conditions and cycle counts were 95 °C for 15 min for initial denaturation, and then 40 cycles of denaturation at 95 °C for15 s, annealing at 60 °C for 60 s, and elongation at 60 °C for 60 s. The real-time PCR efficiencies were determined for target and internal control genes with the slope of a linear regression model [34]. Each cDNA sample was bulked and then used as the PCR template in the range from 2 to 50 ng. The corresponding real-time PCR efficiencies were calculated according to the equation: $E = 10^{[-1/\text{slope}]}$ [35]. For target and internal control genes PCR efficiencies were calculated by measuring the CT to a specific threshold for a serial dilution of bulked cDNA. All PCRs displayed efficiencies between 86% and 94%.

For each group at the 4, 24, 48 and 72 h post irradiation time points three independent blood samples were assessed. For each sample, assays were performed in triplicate. The comparative $2^{-\Delta\Delta CT}$ [36] was used for relative fold changes in expression of target genes (bax and bcl-2), normalized to an endogenous reference (GAPDH gene) and a relevant untreated and unirradiated control. ΔΔCT is the difference between the mean ΔCT (treatment group) and mean ΔCT (control group), where ΔCT is the difference between the mean CT gene of interest and the mean CT internal control gene in each sample.

2.5. Statistical analysis

Each data point represents mean ± SEM of at least three independent experiments 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.

3. Results

The apoptotic lymphocytes analysis, bax and bcl-2 genes relative expression and bax/bcl-2 ratio of eight groups at all time-points are given in Figs. 1–8.

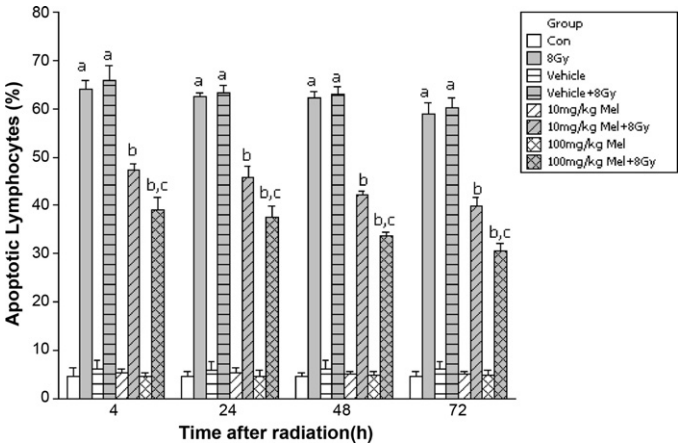

Fig. 2. Effect of melatonin on radiation-induced apoptosis in rats peripheral blood lymphocytes. Rats were exposed to a single whole body γ-ray dose of 8 Gy with or without melatonin (Mel) pretreatments (10 and 100 mg/kg IP 1 h before irradiation). Apoptotic lymphocytes were analyzed by flow cytometric assay 4, 24, 48 and 72 h post irradiation. The percentages of apoptotic lymphocytes were shown in experimental groups. Values are expressed as mean ± SEM of three independent samples. [a] $P < 0.01$ compared to the control group, [b] $P < 0.01$ compared to the 8 Gy and vehicle + 8 Gy groups, [c] $P < 0.01$ compared to the 10 mg/kg Mel + 8 Gy group.

3.1. Apoptotic lymphocytes analysis

As shown in Figs. 1 and 2, at the 4 h post irradiation, 8 Gy gamma exposure caused a marked increase in the percentage of apoptotic lymphocytes (Annexin V^+ and PI^-) as compared with the control group (64.14 ± 1.07% versus 4.75 ± 0.96%; $P < 0.01$). In group 4, administration of vehicle 1 h before gamma irradiation induced the early apoptotic lymphocytes closely related to the gamma radiation alone. However, in the rats treated with 10 mg/kg body weight (B.W.) melatonin 1 h before 8 Gy gamma exposure, the percentage of apoptotic lymphocytes decreased when compared with 8 Gy gamma ray only and vehicle plus radiation groups (47.40 ± 0.73% versus 64.14 ± 1.07% and 65.81 ± 1.84%, respectively; $P < 0.01$). Also, a more reduction in the apoptotic lymphocytes was observed in the rats treated with 100 mg/kg B.W. melatonin 1 h before 8 Gy gamma radiation in comparison with 8 Gy gamma radiation only and vehicle plus radiation groups (39.05 ± 1.47% versus 64.14 ± 1.07% and 65.81 ± 1.84%, respectively; $P < 0.01$). At 24 h after gamma irradiation, flow cytometric analysis shown almost similar results in the population of apoptotic lymphocytes in comparison with 4 h post irradiation in experimental groups (Figs. 2 and 3). As observed in Figs. 2 and 4, at 48 h post irradiation, the irradiated rats that were pre-treated with 10 mg/kg and 100 mg/kg melatonin in groups 6 and 8 exhibited a further decrease in the percentage of apoptotic lymphocytes (42.21 ± 0.38% and 33.60 ± 0.47%, respectively; $P < 0.01$) while the population of apoptotic lymphocytes in 8 Gy gamma radiation only (62.14 ± 0.85%) and vehicle plus radiation groups (62.95 ± 0.93%) were almost similar to same experimental groups at 4 and 24 h after irradiation (Fig. 2). At the 72 h post irradiation, the percentages of apoptotic lymphocytes in experimental groups were similar to results observed at the 48 h following irradiation (Figs. 2 and 5). The significant increase in the population of apoptotic lymphocytes maintained in the irradiated rats with gamma radiation of 8 Gy alone and the rats administered with vehicle before gamma exposure until 72 h post irradiation (64.14 ± 1.07% and 65.81 ± 1.841% at 4 h, 62.38 ± 0.55% and 63.38 ± 0.90% at 24 h, 62.14 ± 0.85% and 62.95 ± 0.93% at 48 h, 58.93 ± 1.31% and 60.07 ± 1.33% at 72 h, respectively; $P < 0.01$). The groups of control, vehicle-only, 10 mg/kg melatonin and 100 mg/kg melatonin alone exhibited similar results in the population of apoptotic lymphocytes at the 4, 24, 48 and 72 h after radiation. Also, there were statistically significant differences in the percentage of apoptotic lymphocytes in the rats treated with 100 mg/kg melatonin before gamma radiation in comparison with those pre-treated with 10 mg/kg melatonin in all time points (39.05 ± 1.47% versus 47.40 ± 0.73% at 4 h, 37.58 ± 1.42% versus 45.67 ± 1.38% at 24 h, 33.60 ± 0.47% versus 42.21 ± 0.38% at 48 h and 30.53 ± 0.97% versus 39.76 ± 1.08% at 72 h, respectively; $P < 0.01$) (Fig. 2). Furthermore, in all different groups, the percentage of necrotic lymphocytes (Annexin V^+ and PI^+) was too low and negligible (<2%) at the indicated intervals following irradiation (Figs. 1, 3–5).

3.2. Bax and bcl-2 genes relative expression and bax/bcl-2 ratio

As shown in Fig. 6, in the irradiation-only group, the expression of bax sharply increased at the initial 4 h relative to controls (13.44 ± 0.53-fold; $P < 0.01$), and remained at the almost similar levels at 24, 48, and 72 h time points after irradiation. In the vehicle plus irradiation group, bax expression was almost similar to irradiation-only group at different time points (Fig. 6). However, in the 10 mg/kg B.W. melatonin pretreatment group, bax expression was significantly downregulated in comparison with irradiation-only and vehicle plus irradiation groups at 4, 24, 48, and 72 h post irradiation (9.65 ± 0.88-fold, 9.37 ± 0.79-fold, 8.66 ± 0.27-fold and 8.35 ± 0.29-fold, respectively; $P < 0.01$) (Fig. 6). Also, a higher reduction in the expression of bax was observed in the 100 mg/kg B.W. melatonin pretreatment group in comparison with irradiation-only and vehicle plus irradiation groups at the indicated intervals following irradiation (8.35 ± 0.30-fold, 8.04 ± 0.40-fold, 7.58 ± 0.41-fold and 7.13 ± 0.46-fold, respectively; $P < 0.01$). The statistically significant differences in the expression of bax was observed only at 48 h and 72 h time points after irradiation in the rats treated with 100 mg/kg melatonin before gamma radiation in comparison with those pre-treated with 10 mg/kg melatonin (7.58 ± 0.41-fold versus 8.66 ± 0.27-fold at 48 h and 7.13 ± 0.46-fold versus 8.35 ± 0.29-fold at 72 h, respectively; $P < 0.05$) (Fig. 6). Furthermore, in the vehicle-only, 10 mg/kg melatonin and 100 mg/kg melatonin alone groups, bax expression was almost similar, 1.11–1.22-fold higher than that in the control group. On the other hand, in the irradiation-only group, the expression of bcl-2 was significantly decreased at the initial 4 h compared to control (0.65 ± 0.03-fold; $P < 0.01$), and sustained at the same level at 24, 48, and 72 h post irradiation. However, in the 10 mg/kg B.W. melatonin pretreatment group, bcl-2 expression significantly upregulated compared to irradiation-only and vehicle plus irradiation groups at 4, 24, 48, and 72 h post irradiation (0.78 ± 0.02-fold, 0.79 ± 0.01-fold, 0.81 ± 0.02-fold and 0.82 ± 0.01-fold, respectively; $P < 0.01$) (Fig. 7). The higher upregulation of bcl-2 was also seen in the rats pretreated with 100 mg/kg B.W. melatonin before gamma exposure in comparison with irradiation-only and vehicle plus irradiation groups at 4, 24, 48, and 72 h post irradiation (0.83 ± 0.02-fold, 0.84 ± 0.01-fold, 0.87 ± 0.01-fold and 0.87 ± 0.02-fold, respectively; $P < 0.01$) (Fig. 7). The statistically significant differences in the expression of bcl-2 was not seen in all time points after irradiation in rats pre-treated with 100 mg/kg melatonin in comparison with those pre-treated with 10 mg/kg melatonin (Fig. 7). This decrease in bax activation and increase in bcl-2 expression resulted in a significantly decreased bax/bcl-2 ratio in the 10 mg/kg melatonin pretreatment groups in comparison with irradiation-only and vehicle plus irradiation groups in all time points (12.30 ± 1.42 versus 20.65 ± 2.02 and 21.07 ± 1.93 at 4 h, 11.82 ± 1.21 versus 20.26 ± 1.54 and 20.53 ± 1.73 at 24 h, 10.62 ± 0.59 versus 19.77 ± 1.92 and 20.22 ± 1.51 at 48 h and 10.15 ± 0.53 versus 19.62 ± 1.66 and 19.46 ± 1.46 at 72 h, respectively; $P < 0.01$) (Fig. 8). Also, pretreatment with 100 mg/kg melatonin decreased bax/bcl-2 ratio in comparison with irradiation-only and vehicle plus irradiation groups in all time points

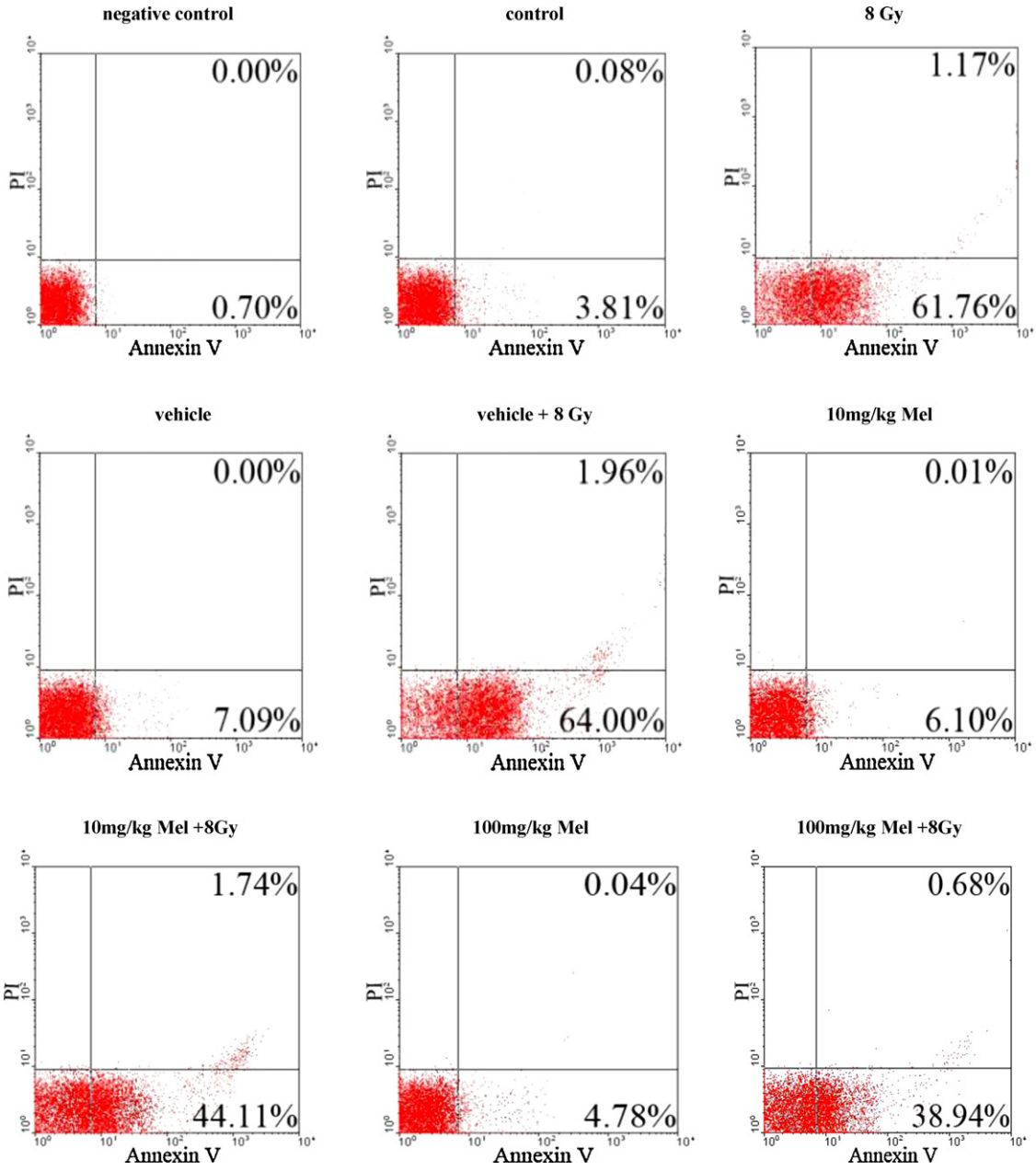

Fig. 3. Effect of melatonin on radiation-induced apoptosis in rats peripheral blood lymphocytes. Rats were exposed to a single whole body γ-ray dose of 8 Gy with or without melatonin (Mel) pretreatments (10 and 100 mg/kg IP 1 h before irradiation). Apoptotic and necrotic lymphocytes were analyzed by flow cytometric assay 24 h post irradiation. Representative dot plots of one set of three independent experiments of Annexin V and PI staining. Apoptotic lymphocytes (Annexin V^+ and PI^-) were displayed in the lower right quadrant and necrotic lymphocytes (Annexin V^+ and PI^+) were shown in the upper right quadrant.

(10.07 ± 0.67 versus 20.65 ± 2.02 and 21.07 ± 1.93 at 4 h, 9.47 ± 0.91 versus 20.26 ± 1.54 and 20.53 ± 1.73 at 24 h, 8.65 ± 0.63 versus 19.77 ± 1.92 and 20.22 ± 1.51 at 48 h and 8.18 ± 0.76 versus 19.62 ± 1.66 and 19.46 ± 1.46 at 72 h, respectively; $P < 0.01$) (Fig. 8). However, the statistically significant differences in bax/bcl-2 ratio was not seen in all time points after irradiation in rats pre-treated with 100 mg/kg melatonin in comparison with those pre-treated with 10 mg/kg melatonin (Fig. 8).

4. Discussion

Because immune cells such as lymphocytes are quite sensitive to ionizing radiation, and that, in many cases, immune depletion appear during the course of anti-cancer treatments [37], immune depletion is one of the serious problems in radiation therapy as well as in accidental whole body exposure to irradiation. Since radiation-induced cellular injury is attributed primarily to the harmful effects of free radicals and free radicals play a key role in irradiation-induced apoptosis [19,20], in the present study, we determined that melatonin, a direct free radical scavenger, remarkably reduces radiation-induced apoptosis in rat lymphocytes. Although, two recent reports have shown that melatonin administration before radiation reduced the frequency of apoptosis and apoptotic index in the splenic lymphocytes of irradiated mice and Indian palm squirrels [38,39], to our knowledge, however, the current in vivo study is the first to demonstrate the molecular mechanism of melatonin for reduction of apoptosis induced by gamma irradiation in rat peripheral blood lymphocytes. In previous investigations, apoptosis of mouse splenic lymphocytes increased following X-ray irradiation in the doses of 2–3 Gy until reaching the highest level at 8–12 h,

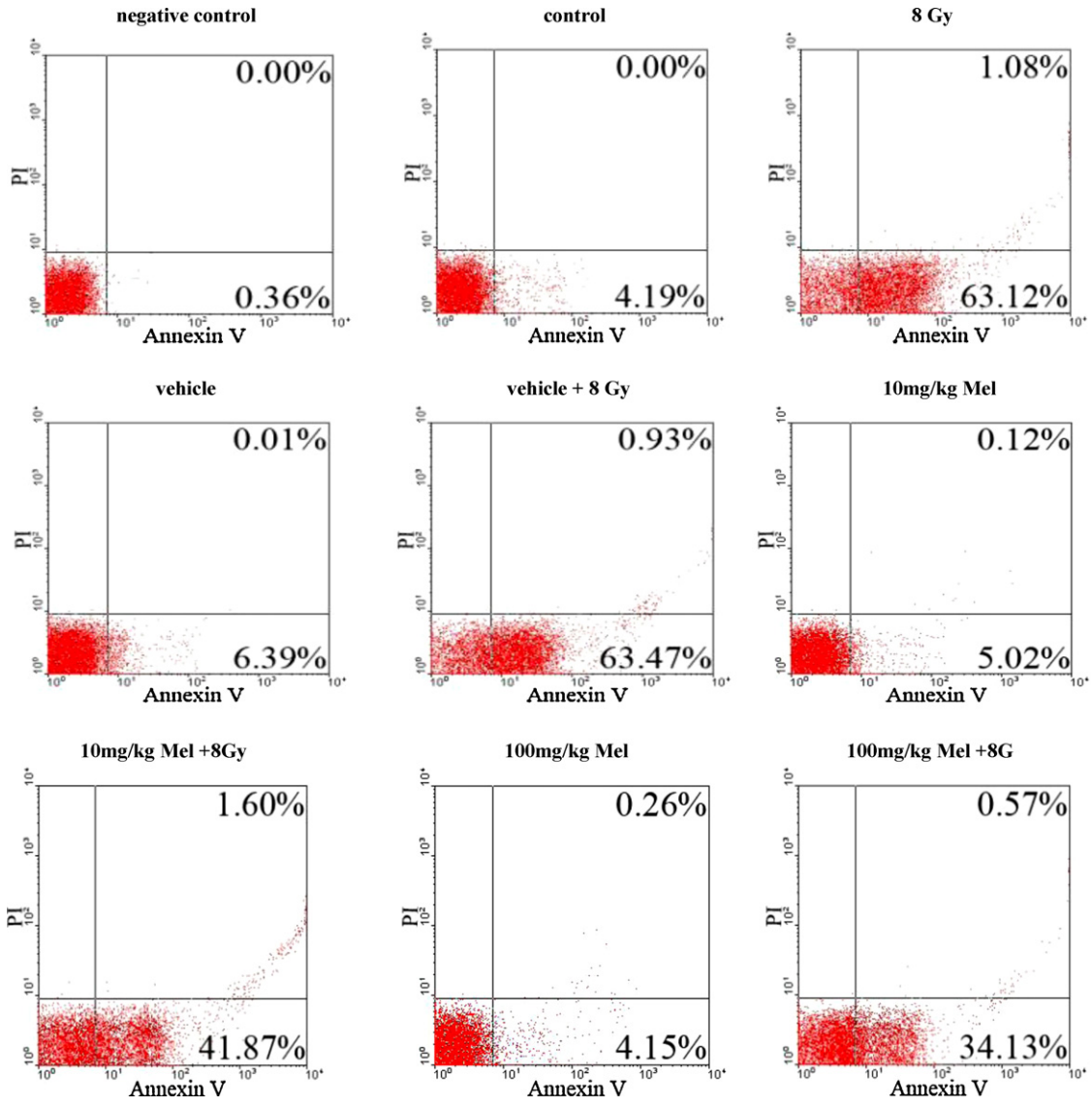

Fig. 4. Effect of melatonin on radiation-induced apoptosis in rats peripheral blood lymphocytes. Rats were exposed to a single whole body γ-ray dose of 8 Gy with or without melatonin (Mel) pretreatments (10 and 100 mg/kg IP 1 h before irradiation). Apoptotic and necrotic lymphocytes were analyzed by flow cytometric assay 48 h post irradiation. Representative dot plots of one set of three independent experiments of Annexin V and PI staining. Apoptotic lymphocytes (Annexin V^+ and PI^-) were displayed in the lower right quadrant and necrotic lymphocytes (Annexin V^+ and PI^+) were shown in the upper right quadrant.

thereafter, apoptosis decreased [15,38]. In our study, 4 h after whole body gamma irradiation of 8 Gy in rats, the population of apoptotic lymphocytes increased rapidly and the significant increase in the percentage of apoptotic lymphocytes maintained in the irradiated rats with gamma radiation of 8 Gy alone and the rats administered with vehicle before gamma exposure until 72 h post irradiation. Our results were in agreement with others [18,37]. In a study, Cui et al. [18] found that 4 h after whole body gamma irradiation at 2–8 Gy in mice, the percentage of mouse peripheral lymphocytes apoptosis increased rapidly and the peak value of circulating lymphocytes apoptosis were on the 7th day after irradiation. Also, they have shown that the up-regulation of bax and down-regulation of bcl-2 expression in irradiated lymphocytes was closely related to apoptosis in peripheral lymphocytes [18]. Furthermore, in another study, Benitez-Bribiesca et al. [37] showed that apoptosis appears only 3 h post-irradiation in human peripheral lymphocytes. In fact, although the process of apoptosis in many cell types and tissues is short-lived, peripheral lymphocytes display an arrested apoptosis after radiation exposure and lymphocytes remain in this state for many days [17]. This is suggesting that lymphocyte apoptosis might be the major cause of lymphocytopenia in the early stage (4th–7th days) after irradiation. In present study, comparison of the apoptosis between the experimental groups in all time points revealed that pretreatment with 10 mg/kg and 100 mg/kg dosages of melatonin significantly reduced the apoptosis induced by gamma irradiation of 8 Gy in rats lymphocytes. However, in another study, Yurtcu et al. [32] declared that 10 mg/kg of melatonin administration immediately before irradiation was unable to reduce radiation-induced apoptosis in cultured rat lymphocytes 48 h after gamma irradiation of 8 Gy. This obvious discrepancy could be attributed to the different methodological procedures employed to identify radiation-induced apoptosis and difference in the time of melatonin administration before radiation. In the present study, Annexin V/PI double staining was used for detection of apoptotic and necrotic lymphocytes while Yurtcu et al. used morphological features such as membrane blebs and apoptotic bodies as apoptosis in rat lymphocytes. One of the earliest hallmarks of apoptosis is the translocation of the phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane and its exposure to the external cellular

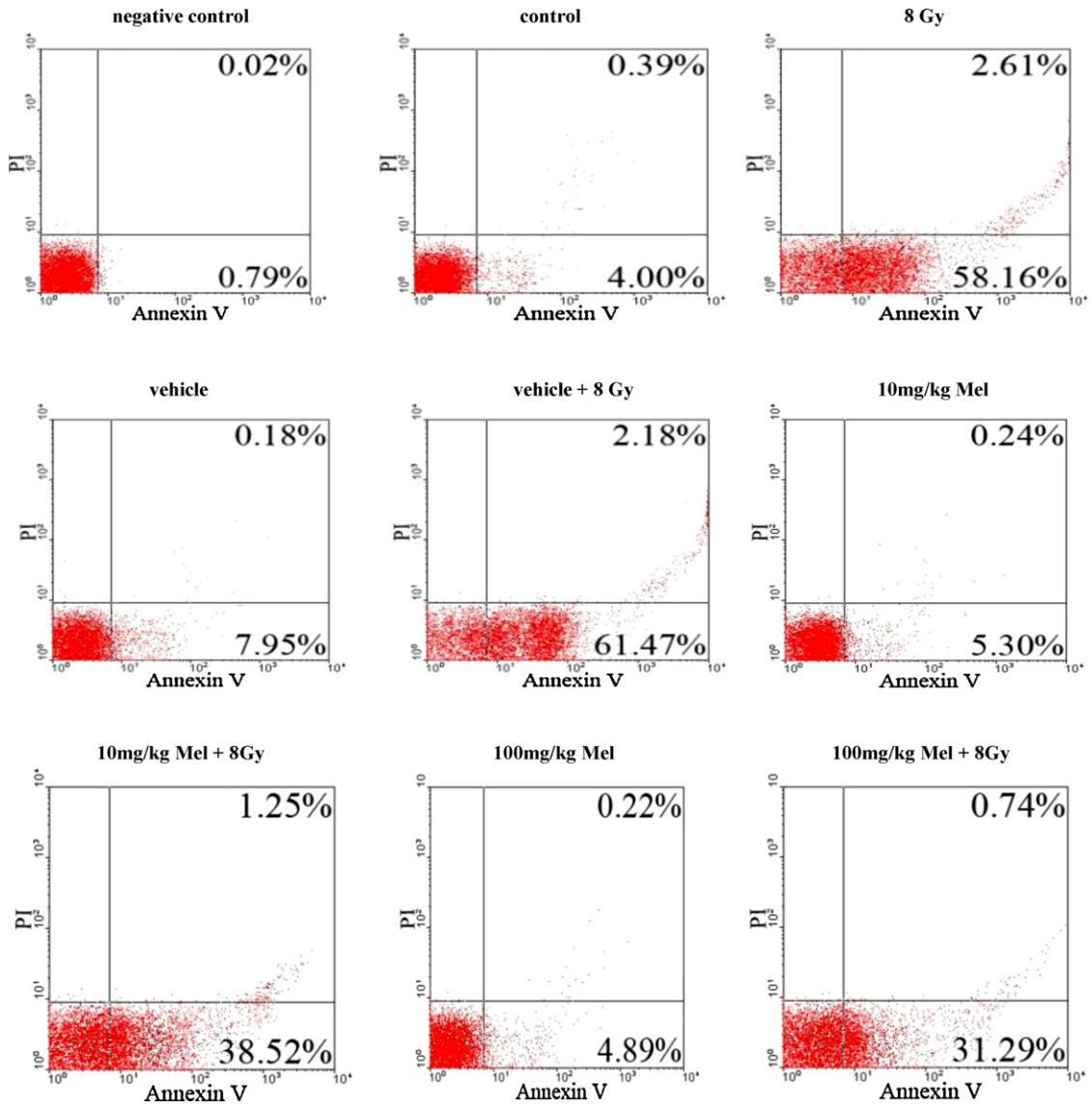

Fig. 5. Effect of melatonin on radiation-induced apoptosis in rats peripheral blood lymphocytes. Rats were exposed to a single whole body γ-ray dose of 8 Gy with or without melatonin (Mel) pretreatments (10 and 100 mg/kg IP 1 h before irradiation). Apoptotic and necrotic lymphocytes were analyzed by flow cytometric assay 72 h post irradiation. Representative dot plots of one set of three independent experiments of Annexin V and PI staining. Apoptotic lymphocytes (Annexin V^+ and PI^-) were displayed in the lower right quadrant and necrotic lymphocytes (Annexin V^+ and PI^+) were shown in the upper right quadrant.

environment. Annexin V, a phospholipid-binding protein, has a high affinity for PS and therefore is known as a sensitive detection method of early stages of apoptosis [40,41]. On the contrary, PI stains the cellular DNA of those cells with advanced cell membrane changes [42]. Therefore, when lymphocytes double-stained with the dyes were analyzed by flow cytometry, four different groups of lymphocytes were differentiated. The live lymphocytes with Annexin V and PI negative staining (Annexin V^- and PI^-), apoptotic lymphocytes with Annexin V positive and PI negative staining (Annexin V^+ and PI^-), necrotic lymphocytes with Annexin V and PI positive staining (Annexin V^+ and PI^+) and necrotic lymphocyte debris or apoptotic bodies with Annexin V negative and PI positive staining (Annexin V^- and PI^+). On the other hand, the time interval between melatonin administration and irradiation of the rats cannot be ignored as another possible explanation for differences between our results and those of Yurtcu et al. [32]. Since it has been proved that radio-protective effects of melatonin occur when melatonin is present in cells and tissues at the time of radiation [19,20], to ensure the presence of melatonin in cells and tissues at the radiation time, 20 min preincubation with melatonin in in vitro studies [27,28], 1–2 h pretreatments with melatonin in in vivo/in vitro investigations [29,43] and 0.5–1 h pretreatments with melatonin in in vivo studies [23,25,26,30,31,38,44,45,49] have been applied in all previous investigations except Yurtcu et al. study. It seems that the lack of radio-protective effect of melatonin on radiation-induced apoptosis in cultured rat lymphocytes may be due to this reason in Yurtcu et al. study. Furthermore, our results showed that gamma radiation-induced lymphocyte death was apoptosis not necrosis in all experimental groups at the indicated intervals following irradiation. Although, it has long been believed that most anti-cancer therapies induce cell necrosis, it is, instead, becoming clear that these treatments induce cell death via apoptosis and that most of these treatments are DNA damaging agents [46,47]. Regarding the close relationship between free radicals, particularly ROS, and apoptosis, this anti-apoptotic effect in our study is supposed to have resulted from the action of melatonin and/or its metabolites as direct free radical scavengers against ROS generated by radiation [21–23,48].

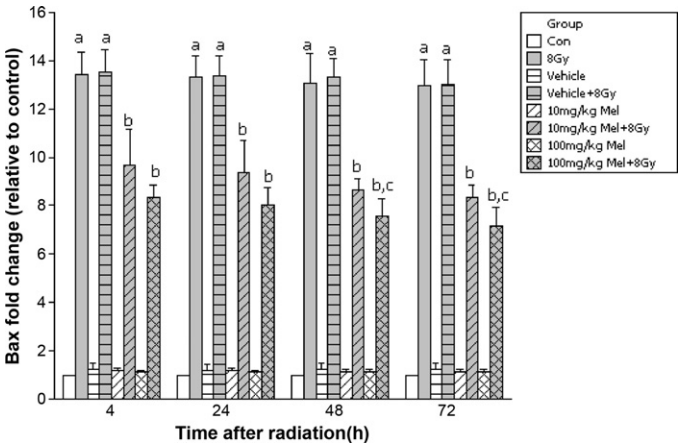

Fig. 6. Real-time quantitative RT-PCR analysis of the fold change of bax at various time points after irradiation (relative to control). Values are expressed as mean ± SEM of three independent samples each performed in triplicate. [a]$P < 0.01$ compared to the control group, [b]$P < 0.01$ compared to the 8 Gy and vehicle + 8 Gy groups, [c]$P < 0.05$ compared to the 10 mg/kg Mel + 8 Gy group.

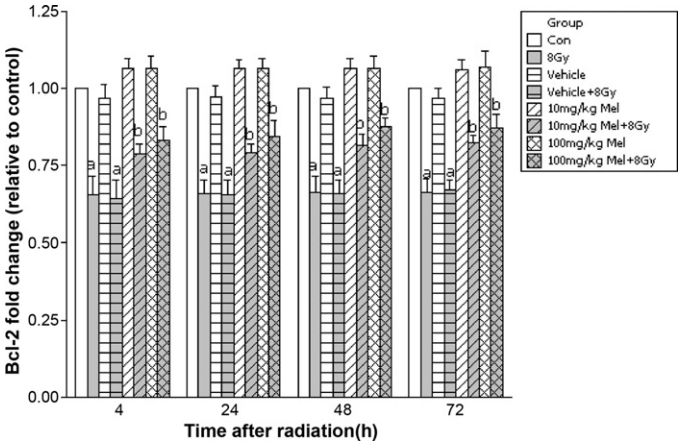

Fig. 7. Real-time quantitative RT-PCR analysis of the fold change of bcl-2 at various time points after irradiation (relative to control). Values are expressed as mean ± SEM of three independent samples each performed in triplicate. [a]$P < 0.01$ compared to the control group, [b]$P < 0.01$ compared to the 8 Gy and vehicle + 8 Gy groups.

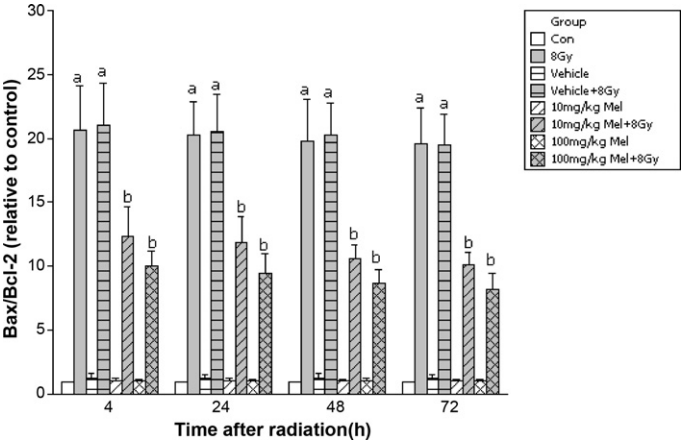

Fig. 8. Real-time quantitative RT-PCR analysis of the fold change of bax/bcl-2 ratio at various time points after irradiation (relative to control). Values are expressed as mean ± SEM of three independent samples each performed in triplicate. [a]$P < 0.01$ compared to the control group, [b]$P < 0.01$ compared to the 8 Gy and vehicle + 8 Gy groups.

The bax and bcl-2 genes are important apoptosis-associated upstream regulators in intrinsic (mitochondrial) apoptotic pathway. The bax and bcl-2 expression levels modify mitochondrial integrity, release cytochrome C and activate caspases. The relative levels of proapoptotic bax against anti-apoptotic bcl-2 may determine the sensitivity of cells to apoptotic stimuli. Thus, the imbalance in the bax/bcl-2 ratio as well as their individual expression level is of great importance [8,9,12–14]. Our study showed that 8 Gy of gamma radiation may produce the upregulation of bax and downregulation of bcl-2 and subsequently increase in bax/bcl-2 ratio in irradiated lymphocytes and it was consistent with the increase in apoptosis in all time points after irradiation, similarly to the results of Cui et al. [18]. Moreover, our findings revealed that 10 mg/kg and 100 mg/kg of melatonin pretreatments reduced the expression of bax at the indicated periods after irradiation and increased the bcl-2 expression in all time points post irradiation. In addition, the significant decrease in the bax/bcl-2 ratio in all time points following irradiation in the 10 mg/kg and 100 mg/kg of melatonin pretreatment groups was consistent with the decrease in apoptosis observed in these groups. Our results suggest that the modulating effect of melatonin on bax and bcl-2 expression levels and consequently the bax/bcl-2 ratio cause the protection of radiation-induced apoptosis in rat lymphocytes. Since in most cases, the bax/bcl-2 ratio ultimately plays a crucial role in determining the apoptosis, the significant decrease in the bax/bcl-2 ratio by melatonin can prevent the translocation of bax from cytoplasm to mitochondria, inhibit opening of mitochondrial permeability transition pores (MPTP) in the mitochondrial membrane, reduce release of cytochrome C to the cytosol and inactivate caspase cascade and finally reduce irradiation-induced apoptosis. Furthermore, our findings show that melatonin at two doses as high as 10 mg/kg and 100 mg/kg are non-toxic, and in a dose-dependent manner, melatonin may significantly decrease bax/bcl-2 ratio and irradiation-induced apoptosis, although these reductions were not in a linear mode.

Our previous study showed that ameliorative effects of melatonin (10 mg/kg) on antioxidant enzymes activity were not statistically significant against deleterious effects of 8 Gy gamma irradiation [49]. While, our present study indicates that rats pretreatment with 10 mg/kg melatonin cause statistically significant changes both in the percentage of apoptotic lymphocytes and apoptotic-related genes expression in all time points post 8 Gy irradiation. It may be due to differential actions of melatonin as a direct free radical scavenger and as an indirect stimulus on antioxidant enzymes activity [48,50,51]. Since the direct free radical scavenging effects of melatonin are independent of any receptor, while its ability to affect on antioxidant enzymes activity probably needs an interaction with either membrane or nuclear receptors [19,52].

5. Conclusion

In conclusion, to the best of our knowledge, this is the first in vivo study to present the molecular mechanism of melatonin that it may be beneficial in reduction of gamma irradiation-induced apoptosis in rat peripheral blood lymphocytes. This study reveals that melatonin in high concentrations may reduce irradiation-induced apoptosis in rat lymphocytes presumably and it may be a modulator of bax and bcl-2 expression levels and bax/bcl-2 ratio.

Observed radio-protective effects of melatonin, suggest that administration of this agent may enable the use of higher doses of irradiation during radiotherapy leading to increase in the therapeutic index. However, further in vivo studies are needed to clarify anti-apoptotic and other properties of this agent.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Source of support

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