Expression Levels of Two DNA Repair-related Genes under 8 Gy Ionizing Radiation and 100 Mg/Kg Melatonin Delivery In Rat Peripheral Blood

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ABSTRACT

Background: After radiation therapy (RT), some health hazards including DNA damages may occur where melatonin can play a protective role due to free radical generation. On the other hand, serious accidental overexposures may occur during RT due to nuclear accidents which necessitate the need for study on exposure to high-dose radiations during treatments.

Objective: The aim of this study was to study the expression level of two genes in non-homologous end joining (NHEJ) pathways named Xrcc4 and Xrcc6 (Ku70) in order to examine the effect of melatonin on repair of DNA double-strand breaks (BSBs) caused by 8Gy ionizing radiation.

Methods: One hundred eight male Wistar rats were irradiated with a whole body gamma radiation dose of 8Gy with or without melatonin pretreatments. They were divided into six different groups of control, 100 mg/kg melatonin alone, 8Gy irradiation alone, vehicle alone, vehicle plus 8Gy irradiation and 100 mg/kg melatonin plus 8Gy irradiation. Peripheral blood samples were collected at 8, 24 and 48 h after irradiation. Ku70 and Xrcc4 gene expression were evaluated by real-time quantitative polymerase chain reaction (qPCR) technique and analyzed by one-way ANOVA test.

Results: Expression of Ku70 and Xrcc4 genes normalized against Hprt gene showed significant difference in melatonin plus irradiation group at 8h compared to the control group ($p<0.05$). At 24h post irradiation, gene expression changes were significantly upregulated in irradiation-alone group as well as melatonin plus irradiation group ($p<0.05$). No significant change was found in any groups compared to control group at 48 h time point.

Conclusion: We concluded that, by increasing expression level of Ku70 and Xrcc4 genes, 100 mg/kg melatonin administration 8 and 24 h before 8 Gyionizing radiation can significantly affect the repair of DNA DSBs in NHEJ pathway.

Keywords

Ionizing Radiation, Melatonin, Gene Expression Level, Ku70, Xrcc4

Introduction

Radiation therapy (RT) is an important treatment modality for cancer patients, but various health hazards may occur after RT [1] such as mutation, radiation sickness, cancer and death. Ionizing radiation may cause DNA damages such as single-strand breaks

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(SSBs), double-strand breaks (DSBs), base and sugar damage, DNA-DNA cross-links and DNA-protein cross-links. Among these, according to many studies such as [2-5], DSB is the most serious type of DNA damage after RT leading to cells death, mutation or carcinogen. It should be repaired to protect the genome and cells survival [6,7]. DNA damages are mainly repaired by non-homologous end joining (NHEJ) pathways [8]. According to Mao et al. [9], NHEJ is a faster and more efficient DSB repair pathway than homologous recombination in human cells. Guirouilh-Barbat et al. [10] suggested that NHEJ is the predominant DSB repair pathway in mammalian cells. NHEJ modifies the broken DNA ends, and ligates them together with no regard for homology, generating deletions or insertions [11]. The proteins which participate in NHEJ pathway include XRCC4, XRCC5 (Ku80), XRCC6 (Ku70), DNA-PKcs, DNA ligase IV, Artemis and XLF [12, 13]. First, both ends of the break are joined by the Ku70/80 heterodimer which protects the DNA ends from degradation. Then, Ku70/80 recruits the catalytic subunit of the DNA dependent protein kinase (DNA-PKcs) to DNA ends to form the active DNA-PK; the ends can be trimmed or filled in by nucleases and polymerases. Finally, the DNA-PKcs complex stimulate the end processing for subsequent ligation by XRCC4/ DNA ligase IV [1, 14-16]. "XRCC5/XRCC6 directly mediates incorporation of XRCC4 into end-joining complexes, and they are the core of the NHEJ reaction" [1].

It has been shown that some radioprotectors can protect cells against ionizing radiation (IR) induced damages by scavenging free radicals. One of the protective agents is melatonin (N-acetyl-5-methoxytryptamine) [17-20]. Many in-vitro and in-vivo studies have shown its favorable efficacy in protecting animal and human cells from the toxic effects of ionizing radiation [21-24], but there is a serious lack of information about its role in DNA repair in the NHEJ pathway. Due to the damage caused IR on living organisms which in high doses can even cause cell death; it is important to protect humans against radiation. On the other hand, serious accidental overexposures involving patients who are undergoing post-operative radiotherapy may occur due to nuclear accidents which necessitates the need for study on exposure to high-dose radiations during treatments (>8Gy). After accidental exposure to high-dose radiation, casualties generally suffer from fatal damage to multiple organs, injuries that are termed acute radiation syndrome, causing severe pancytopenia and fatal immune dysfunction [25]. In this regard, in this study, we investigated the DNA DSBs induced by 8Gy irradiation in rat peripheral blood. For this purpose, we examined the expression level of Ku70 and Xrcc4 genes involved in 8Gy whole-body gamma irradiation and 100 mg/kg melatonin delivery to study the effect of melatonin on the repair of DNA DSBs caused by high-dose radiations.

Material and Methods

Animals, Experimental Design and Irradiation

All experiments were in accordance with the guidelines for care and use of laboratory animals adopted by the Ethics Committee of Tehran University of Medical Sciences (TUMS), Tehran, Iran. Animals were one hundred eight 70-day old male Wistar rats provided from pharmacy faulty of TUMS with a body weight range of 180 to 220 g. They were kept in a room temperature and maintained at 20-22ºC and light-controlled environment with a 12 hours of darkness/ 12 hours of light cycle. After one week acclimatization period, animals were randomly divided into six different groups: 1- Control (CON), 2- Melatonin alone (MEL), 3- Irradiation alone (IR), 4- Vehicle (VEH), 5- Vehicle + irradiation (VEH+IR), and 6- Melatonin + irradiation (MEL+IR). In the first group, rates received no melatonin or irradiation but received both an intra-peritone-

al (IP) injection of 500 µl of phosphate-buffered saline (PBS) and sham-irradiation; in the second group, all anesthetized rats received 100 mg/kg melatonin with IP injection of 500 µl PBS and final ethanol concentration 5% one hour before irradiation, and then they went under-sham irradiation. It should be noted that all rats were anesthetized with an IP injection of ketamin (100 mg/kg) and xylazin (5 mg/kg); in group 3, rats went under 8Gy whole-body gamma radiation and received the same volume of PBS 1 h before irradiation; in group 4, rats received 5% absolute ethanol with IP injection of 500 µl PBS; in group 5, rats received 5% absolute ethanol with IP injection of 500 µl PBS plus 8Gy whole-body gamma radiation; and finally, in group 6, rats received 100 mg/kg melatonin with 5% absolute ethanol and an IP injection of 500 µl PBS plus 8Gy whole-body gamma radiation. It should be mentioned that melatonin first was dissolved in a small amount of absolute ethanol (25µl) and then diluted by PBS (475µl) in 5% final ethanol concentration according to previous studies such as [26]. All rats were anesthetized with ketamin (100 mg/kg) and xylazin (5 mg/kg) by an IP injection before any intervention based on previous studies like [27]. A 6 MV X-ray linear accelerator machine (Elekta Compact 6 MV, China) with a fixed field size of 35×35cm was used for irradiation of rats at room temperature (22 ± 2ºC). To ensure the output of the accelerator, before irradiation, dosimetry and calibration were performed by using an ionizing chamber based on International Atomic Energy Agency (IAEA) TRS-398 standard.

Blood Sample Preparation

There were 18 rats in each study group divided in three subgroups containing six rats. Peripheral blood samples were taken from all of these subgroups on EDTA sterile tubes at 8, 24 and 48h after irradiation. Hybrid-R blood RNA mini 315-150 kit (Gene All Biotechnology, Seoul, South Korea) was used for isolating total RNA from whole blood according to the manufacturer's instructions. Also, a Nanodrop-2000 spectrophotometer (Thermo Scientific, Wilmington, USA) was used for quantifying the extracted RNA at 260/280 nm ratio and its purity was quantified by the spectrophotometer at 260/230 nm ratio. The integrity of isolated RNA was confirmed with Agarose gel electrophoresis. The obtained samples were stained with ethidium bromide to visualize the 18S and 28S RNA subunits by band size discrimination under UV transillumination. For synthesis of cDNA, a 2-µg aliquot of the total RNA was reversely transcribed in a total volume of 20µl by using Hyperscript TM first strand synthesis Kit (GeneAll Biotechnology, Seoul, South Korea).

Real-time Quantitative RT-PCR Analysis

Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) was applied to study the expression of Ku70 and Xrcc4 genes. The device for PCR application was the real-time PCR as performed by Rotor-Gene SYBR Green PCR kit (QIAGEN) using SYBR Premix Ex Taq kit No.RR820L (TaKaRa) on Corbett Rotor-Gene 6000 (Corbett Research, Sydney, Australia) according to the manufacturer's instructions. After RNA isolation and cDNA synthesis, Ku70 and Xrcc4 primers were designed by using Gene Runner software and their expression were determined by using Hprt as an internal control. The sequences of forward and reverse primers are as following:

- Ku70: forward primer = GCT TGT CTT CCT CCC TTA CG, reverse primer = CGA AAC TGT CGC TCC TGT ATG;
- Xrcc4: forward primer = CTG AGG AGG ATG GGC TTT ATG AT, reverse primer = CAA GAT TTG TCT GCA TTC GGT GT;
- Hprt: forward primer = CCA GTC AAC GGG GGA CAT AAA, reverse primer = GGG GCT GTA CTG CTT GAC CAA.

To verify primer specificity in the absence

of DNA amplification, Basic Local Alignment Search Tool (BLAST) was used. The primers were synthesized by Takapou Zist Company in Iran.

The PCR had three cycling conditions: (a) initial denaturation at 95ºC for 10 min followed by (b) cycles of denaturation at 95ºC for 10s and (c) annealing at 60ºC for 20s. Since relative quantification or relative gene expression is the parameter used for relative fold changes in expression of Ku70 and Xrcc4 genes (target genes) normalized to an internal reference (Hprt gene), several data analysis procedures have been developed. One of the more generalized methods is ΔΔCT model [28]. In this method, the ratio of target gene in treated sample relative to untreated sample is calculated as:

$$Ratio=2^{-\Delta\Delta CT} \quad (1)$$

Where,

$\Delta\Delta CT = \Delta CT$ (*treatment sample*) - ΔCT (*control sample*)

ΔCT (*treatment sample*) = *CT* (*target gene*) - *CT* (*reference gene*)

ΔCT (*control sample*) = *CT* (*target gene*) - *CT* (*reference gene*)

In above equations, CT is the threshold cycle generated by the qPCR system, i.e. the cycle number at which the PCR product crosses the threshold. For each group at 8, 24 and 48h post-irradiation time points, six independent blood samples were assessed. Assays were performed in duplicate for each sample.

Statistical Analysis

All data were expressed in terms of mean ± standard error of the mean (SEM) for each group and analyzed by one-way ANOVA test. A value of $P<0.05$ was considered statistically significant.

Results

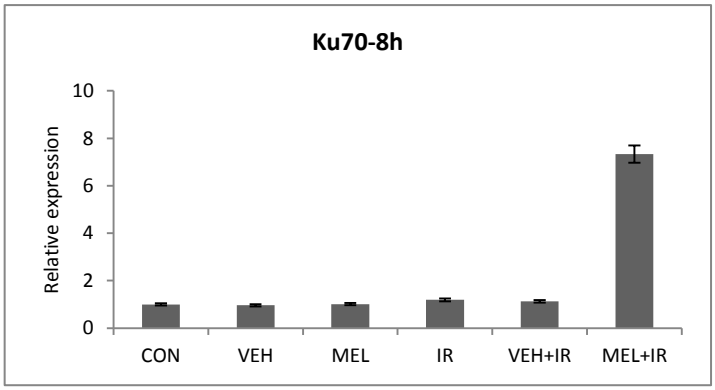
The expression of Ku70 and Xrcc4 genes (normalized against Hprt) in the rat peripheral blood after at 8, 24 and 48 h after irradiation of lethal dose of 8Gy were examined by real-time PCR. Following, we present the real-time PCR results. Moreover, we present the effect of 100 mg/kg melatonin delivery on the expression of Ku70 and Xrcc4 genes.

Expression Level of Ku70

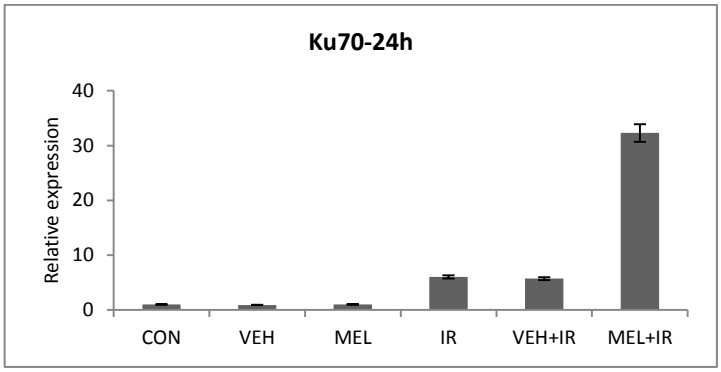
The results revealed that in comparison with the control group, expression changes of Ku70 gene after 8h post-irradiation differs significantly only in MEL+IR group which was 26.58 ±0.39 versus Hprt gene expression changes as 23.95 ± 0.22($p<0.05$). In CON, VEH, MEL, IR and VEH+IR groups, it was 28.36 ± 0.19vs. 22.85 ± 0.15, 29.15 ±0.31 vs. 23.59 ± 0.32, 29.31 ± 0.25 vs. 23.83 ± 0.21, 28.05 ± 0.49 vs. 22.80 ± 029, and 28.73 ± 0.28 vs. 23.40 ± 0.21, respectively (see Figure 1a). After 24h, Ku70 gene expression changes showed a significant difference only in groups IR and MEL+IR which were 31.14 ± 0.10 versus 27.01 ± 0.33 and 26.82 ±0.23 versus 25.11 ± 0.27, respectively ($p<0.05$). For other groups, we had expression changes as following: CON, 29.71 ± 0.57 vs. 22.99 ± 0.40; VEH, 31.80 ± 0.37 vs. 24.97 ± 0.18; MEL, 30 ± 0.50 vs. 23.32 ± 0.30; VEH+IR, 30.57 ± 0.24 vs. 26.36 ± 025 (see Figure 1b). After 48h post irradiation, no significant difference was found in Ku70 gene expression in treatment groups compared with the control group (CON, 28.38± 0.25 vs. 23.28 ± 0.13; VEH, 28.8 ± 0.22 vs. 23.59 ± 0.32; MEL, 28.2 ± 0.21 vs. 23.16 ± 0.14; IR, 31.6 ± 022 vs. 25.86 ± 0.26; VEH+ IR, 29.69 ± 0.25 vs. 24.18 ± 0.27; MEL+IR, 26.88 ± 0.17 vs.21.84 ± 0.24) ($p>0.05$; see Figure 1c).

Expression Level of Xrcc4

Results of statistical analysis for Xrcc4 gene revealed that in a period of 8 hours after irradiation, Xrcc4 gene expression changes versus Hprt gene expression changes were significantly different only in group MEL+IR compared with the control group (25.64 ± 0.15; $p<0.05$). For CON, VEH, MEL, IR and VEH+IR groups, Xrcc4 gene expression changes were 27.60± 0.24, 28.37±0.27, 28.48± 0.78, 26.64±



(a)



(b)

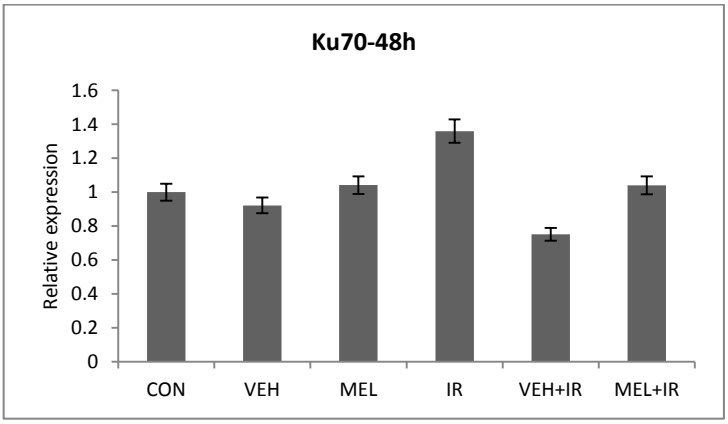
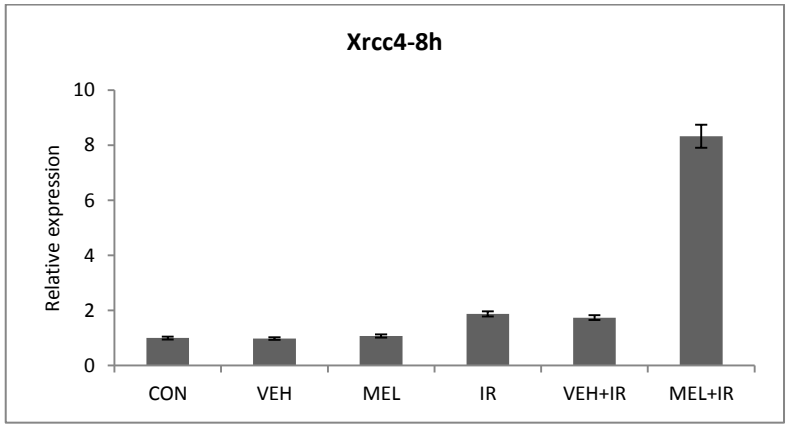


Figure 1: Expression levels of Ku70 in rats' peripheral blood (a) 8, (b) 24 and (c) 48h after 8 Gy-radiation. The columns represent the mean value ± SEM for the six study groups

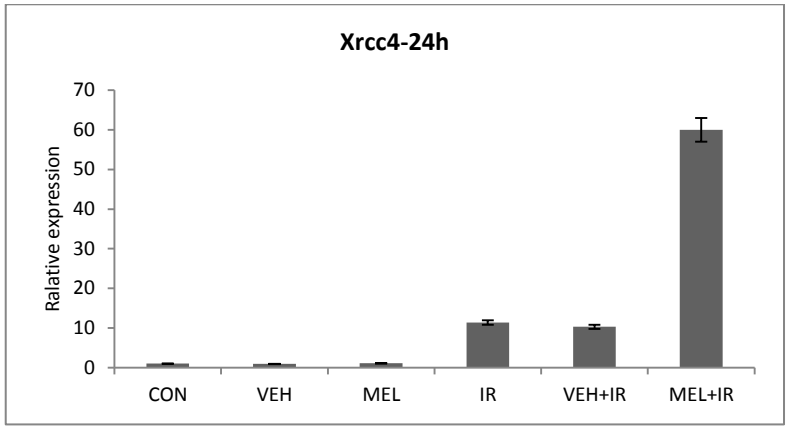
0.42, and 27.35± 0.33, respectively (see Figure 2a). After 24h, Xrcc4 gene expression changes were significantly different only in groups IR and MEL+IR which were 30.23 ± 0.20 and 25.93 ± 0.19, respectively ($p<0.05$; see Figure 2b). At 48h time point, we observed no significant changes in Xrcc4 gene expression (CON, 28.29± 0.36; VEH, 28.68 ± 023; MEL, 28.03 ± 0.22; IR, 30.46 ± 00.18; VEH+ IR, 28.84 ± 0.25; MEL+IR, 25.74 ± 0.20) ($p<0.05$; see Figure 2c).

Effect of Melatonin on Expression Level of Ku70 and Xrcc4

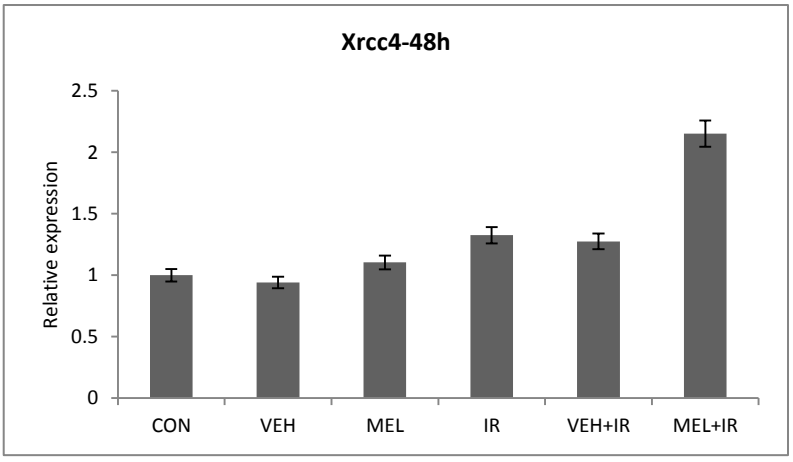
In order to identify the effects of 100 mg/kg melatonin on the expression of genes studied



(a)



(b)



(c)

Figure 2: Expression levels of Xrcc4 in rats' peripheral blood (a) 8, (b) 24 and (c) 48h after 8 Gy-radiation. The columns represent the mean value ± SEM for the six study groups

in this study, their relative expression ratio of 8Gy radiation plus melatonin administration to 8Gy radiation alone was calculated. The results of this ratio reveal a multiplier effect of melatonin on the expression of these genes and thus, the effect of melatonin in the pro-

cess of repairing DNA double-stranded breaks by NHEJ pathway method. According to the results presented in Table 1, Ku70 gene expression level with 8Gy whole-body gamma irradiation and100 mg/kg melatonin injection before irradiation at 8, 24, and 48hpost-irradiation time points were 6.13, 5.36, and 0.76, respectively. These results for Xrcc4 gene were 4.44, 11.39, and 1.62, respectively.

Discussion

There are many studies conducted on the protective role of melatonin against DNA damages. For example, Shirazi et al. [29], Mohseni et al. [30], and Rezaeejam et al. [31] studied the effect of melatonin on apoptosis and showed that melatonin decreases relative expression of pro-apoptotic Bax and increases anti-apoptotic Bcl-2 genes. The current study investigated the effect of 100 mg/kg melatonin on repair of DNA double-stranded breaks under 8Gy radiation in rats. Karbownik et al. [32] examined the potential protective effect of melatonin against 8Gy whole-body radiation and found that melatonin completely counteracted the effects of ionizing radiation. The melatonin concentration was selected according to previous studies such as [29-31, 33] who showed that this concentration does not have any toxicity. Kim and Lee [34] revealed that in the group pretreated with 100 µg of melatonin before irradiation, the ratio of normal primordial follicles was significantly higher than that of the irradiation group at any time point after irradiation. Undeğer et al. [18] found out that the pretreatment of rats with intraperitoneal doses of 100 mg/kg melatonin provided a significant decrease in the DNA strand breakage and lipid peroxidation. In another study by El-Missiry et al. [35], the administration of 100 mg/kg melatonin alone daily for 4 days caused significant decreases in malondialdehyde and protein carbonyl content. Their results showed radioprotective impact of melatonin against ionizing-radiation-induced oxidative stress and organ injury.

In the current study in order to examine the effect of 100 mg/kg melatonin, we investigated the expression level of Ku70 and Xrcc4 genes which are the core of DNA DSBs repair-related genes [12, 13]. In a similar study, Zhang et al. [1] studied the effects of expression level of Ku70, Ku80, and Xrcc4 genes on radiation-induced cognitive impairment in rats NHEJ pathway method using Real-time PCR. Their results showed that receiving 20Gy irra-

Table 1: Multiplier effect of melatonin on the expression of Ku70 and Xrcc4 genes under 8Gy radiation

	8h			24h			48h		
	Gene expression change in MEL+IR group	Gene expression change in IR group	Relative expression Ratio	Gene expression change in MEL+IR group	Gene expression change in IR group	Relative expression Ratio	Gene expression change in MEL+IR group	Gene expression change in IR group	Relative expression Ratio
Ku70	7.36	1.20	6.13	32.29	6.02	5.36	1.04	1.36	0.76
Xrcc4	8.34	1.88	4.44	60	11.39	5.27	2.16	1.33	1.62

diation revealed poorer learning and memory, while no significant loss of learning and memory existed in rats receiving irradiation from 0-10 Gy. Moreover, they found no significant difference in the expression level of Ku70, Ku80, and Xrcc4 genes between the 10 and 20 Gy groups. According to them, DNA damage caused by 0–10Gy exposure was appropriately repaired. In our study, at 8h time point, real-time PCR results indicated that vehicle and melatonin supplementation as well as 8Gy radiation alone had no effect on expression of Ku70 and Xrcc4 genes, but if melatonin was injected before irradiation with 8Gy dose, a significant increase in the expression of these genes would be possible. At 24h time point, results showed that vehicle and melatonin injection alone does not affect Ku70 and Xrcc4 gene expression level, but with 8Gy radiation alone as well as melatonin injection plus 8Gy radiation expression level of Xrcc4 and Ku70 genes increases. According to the results, since changes in Xrcc4 and Ku70 genes expression in MEL+IR group was higher compared to IR group after 24h, we suggest that melatonin injection plus irradiation with 8Gy dose can significantly increase their expression level more than when there is only treatment with 8Gy radiation. Finally, we found out that the injection of 100 mg/kg melatonin before irradiation with 8Gy dose after 48 hours had no significant effect on increase of Ku70 and Xrcc4 genes expression changes. By comparing relative expression changes of Ku70 with Xrcc4 gene, we found out that the greatest effect of 100 mg/kg melatonin on the expression change of Ku70 gene was at 8h time point, while for Xrcc4 gene, it was at 24h time point. Overall, results illustrated that DSB damages were repaired at 24 h and there was no repair at 8 and 48 h after irradiation in the NHEJ pathway, while the DSBs were repaired at 8 and 24 h post-irradiation when melatonin was injected before irradiation. The repair process was also accelerated and strengthened in this condition at 24 h post-irradiation. On the other hand, melatonin also had no effect on repair at 48 h after irradiation.

Conclusion

According to the results, we concluded that the administration of 100 mg/kg melatonin 8 and 24 h before 8Gy ionizing radiation can significantly increase repair of DNA double-stranded breaks in NHEJ pathway by increasing expression level of Ku70 and Xrcc4 genes. We suggest further investigation in terms of different radiation doses, post-irradiation times and DNA repair genes.

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Conflict of Interest

None

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