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Effects of melatonin on repair of DNA double strand breaks caused by ionizing radiation in rat peripheral blood

Majid Valizadeh[1], Alireza Shirazi*[2], Pantea Izadi[3] and Javad Tavakkoli Bazzaz[4], Hamed Rezaeejam[5] and Ghasem Azizi Tabesh[6]

[1,2,5]*Department of Medical, Physics and Biomedical Engineering, Tehran University of Medical Sciences, Tehran, Iran*

[3,4,6]*Department of Medical Genetics, Tehran University of Medical Sciences, Tehran, Iran*

ABSTRACT

The present study aimed to evaluate the effect of melatonin on DNA Double-Strand Breaks repair using gene expression change of Ku70 and Xrcc4 in rat peripheral blood. One hundred eight male rats were randomly divided in six different groups of control, vehicle-only, melatonin alone, irradiation-only, vehicle plus irradiation, and melatonin plus irradiation. Rats were given an intraperitoneal (IP) injection of melatonin (100 mg/kg) 1 hr prior to irradiation. Irradiation was done with 2Gy whole-body radiation by linear accelerator. Peripheral blood samples were collected at 8, 24 and 48 h after irradiation and then RNA was immediately extracted from the peripheral blood lymphocytes and the mRNA transcriptional changes of Ku70 and XRCC4 were evaluated by real-time quantitative polymerase chain reaction (Real-time PCR). Irradiation-only group showed an increase in gene expression change of Ku70 and XRCC4, which was significantly different from control group ($P<0.01$) in 24 h post-irradiation, while in melatonin plus irradiation group, Ku70 and XRCC4 genes were upregulated significantly compared to control group ($P<0.01$) at all three post-irradiation times. It is concluded that melatonin may provide modulation of Ku70 and XRCC4 expression to protect rat peripheral blood lymphocytes against ionizing radiation.

KEY WORDS: IONIZING RADIATION, DOUBLE-STRAND BREAK, MELATONIN, DNA REPAIR

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**Corresponding Author: shirazia@tums.ac.ir*

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INTRODUCTION

Ionizing radiation (IR) is applied constructively in a wide variety of fields such as medicine, research, manufacturing, construction especially in medical diagnostic for cancer treatment (Jobert et al. 2011; Thoms and Bristow, 2010), but presents a health hazard if proper measures against undesired exposure aren't followed. Exposure to ionizing radiation causes damage to living tissue, and can result in mutation, radiation sickness, cancer, and death. The absorption of ionizing radiation by living cells can directly disrupt atomic structures, producing chemical and biological changes. It can also act indirectly through radiolysis of water, thereby generating reactive chemical species that may damage nucleic acids, proteins and lipids. DNA damage of exposed tumour tissue leading to cell death is one of the detrimental effects of ionizing radiations, (Hall and Giaccia, 2006, Azzam et al.2012; Lomax et al. 2013 and Rezaeejam et al 2015).

There are many different forms of IR-induced DNA damages such as single-strand breaks (SSBs), double-strand breaks (DSBs), base and sugar damage, DNA-DNA cross-links and DNA-protein cross-links. DSBs are lesions formed when both strands of the DNA duplex are broken. DSBs are highly toxic and are the most important IR-induced DNA damages in chromosomes after exposure which leads to cells death, mutation or carcinogen. They must be repaired to protect the genome and cells survival, (Brandsma and Gent, 2012; Ciccia and Elledge, 2010 Chapman et al. 2012).

These kinds of damages are mainly repaired by homologous recombination (HR) and non-homologous end joining (NHEJ) pathways (Lieber, 2010). HR leads to accurate repair, while NHEJ is intrinsically mutagenic. Mao et al (2008) suggested that NHEJ is a faster and more efficient DSB repair pathway than HR in human cells. According to Guirouilh-Barbat et al. (2004), NHEJ is the predominant DSB repair pathway in mammalian cells. NHEJ is the major DSB repair pathway in eukaryotes and is utilized in the cellular response of mammalian cells to the repair of IR-induced DSBs. NHEJ can take place throughout the cell cycle. NHEJ modifies the broken DNA ends, and ligates them together with no regard for homology, generating deletions or insertions (Lieber, 2008), while HR uses an undamaged DNA template to repair the break, leading to the reconstitution of the original sequence (Thompson and Schild, 2001).

The proteins that participate in NHEJ pathway include XRCC4, XRCC5 (Ku80), XRCC6 (Ku70), DNA-PKcs, DNA ligase IV, Artemis, and XLF (Bassing et al. 2002; Shrivastav et al. 2008). 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 stimulates the end processing for subsequent ligation by XRCC4/DNA ligase IV (Ahnesorg et al. 2006; San Filippo et al.2008; Schulte-Uentrop et al. 2008; Zhang et al. 2013).

The radio protective agents protects against the damaging effects of IR with various mechanisms. Many studies have reported the ability of melatonin (N-acetyl-5-methoxytryptamine), a pineal gland hormone, to protect against IR-induced damages (Reiter, 1991; Undeger et al. 2004; Shirazi et al. 2011; Shirazi, 2011). It scavengers free radicals, directly and indirectly, especially the highly toxic hydroxyl radicals. Melatonin is also an antioxidant agent by increasing antioxidant enzyme activity and inhibiting pro-oxidative enzyme activity (Koc et al. 2003; Rodriguez et al. 2004; Parihar et al. 2007). Many in vitro and in vivo investigations have confirmed that melatonin protects mammalian cells from the toxic effects of ionizing radiation. Furthermore, several clinical reports indicate that melatonin administration, either alone or in combination with traditional radiotherapy, results in a favorable efficacy (toxicity ratio) during the treatment of human cancers (Vijayalaxmi et al. 2004).

There are many studies which have examined melatonin as a radio protector in radiobiology but there is a serious lack of information about its impact on the DNA repair with NHEJ pathway. In this regard, in this study we have investigated the effect of melatonin on the repair of IR-induced DNA DSB in the peripheral blood of rat when NHEJ pathway is used. We studied the expression change of Ku70 and XRCC4 genes under 2Gy whole-body gamma irradiations to show the radioprotective effect of 100 mg/kg administered melatonin on DNA DSB repair.

MATERIALS AND METHOD

In this *in vivo* study, all experiments were 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 (TUMS), Tehran, Iran. One hundred eight 70-day old male Wistar rats with a body weight range of 180 to 220 g were used for the study from pharmacy faulty of TUMS. They were kept in a room temperature and maintained at 20-22° C and light-controlled environment with a 12-hour light/dark cycle. All rats were given standard diet with no additives.

EXPERIMENTAL DESIGN AND IRRADIATION

After one week acclimatization period, animals were randomly divided into six different groups:

Group 1. Control (CON): In this group, rates received no melatonin or irradiation but received both an intraperitoneal (IP) injection of 500 µl of phosphate-buffered saline (PBS) and sham-irradiation;
Group 2. Melatonin (MEL): In this group, one hour before irradiation, all anesthetized rats received 100 mg/kg melatonin with IP injection of 500 µl PBS and final ethanol concentration 5% and then they went under and 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);
Group 3. Irradiation (IR): In this group rats went under 2 Gy whole body gamma radiation and received the same volume of PBS 1 h prior to irradiation;
Group 4. Vehicle (VEH): In this group rats received 5% absolute ethanol with IP injection of 500 µl PBS;
Group 5. Vehicle + irradiation (VEH+IR): In this group rats received 5% absolute ethanol with IP injection of 500 µl PBS plus 2 Gy whole body gamma radiation;
Group 6. Melatonin + irradiation (MEL+IR): In this group rats received 100 mg/kg melatonin with 5% absolute ethanol and an IP injection of 500 µl PBS plus 2 Gy 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 final ethanol concentration 5% based on previous studies like Cassatt et al. (2002). Also, 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 Prasad (1995). Rats were irradiated with a 6 MV X-ray linear accelerator machine (Elekta Compact 6 MV, China) with a fixed field size of 35cm×35cm at room temperature (22 ± 2°C). Before irradiation, to ensure the output of the accelerator, dosimetry and calibration were performed by using an ionizing chamber based on International Atomic Energy Agency (IAEA) TRS-398 standard.

BLOOD SAMPLE PREPARATION, RNA ISOLATION AND CDNA SYNTHESIS

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

QUANTITATIVE REAL TIME RT-PCR

Quantitative real time PCR was used to measure the expression of Ku70 and XRCC4 genes. After RNA isolation and cDNA synthesis, Ku70 and XRCC4 primers were designed by Gene Runner software and their expression were determined by using HPRT as an internal control. The sequences of forward and reverse primers were as follows: 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; and HPRT, forward primer: CCA GTC AAC GGG GGA CAT AAA, reverse primer: GGG GCT GTA CTG CTT GAC CAA. Basic Local Alignment Search Tool (BLAST) searches were also conducted to verify primer specificity in the absence of DNA amplification. The primers were synthesized by Takapouzist laboratory in Tehran, Iran.

The real time polymerase chain reactions (real time PCR) were carried out by the Rotor-gene Q system (QIAGEN), based on the SYBR green method using the SYBR Premix Ex Taq No. RR820L (TaKaRa) following the manufacturer's instructions. The PCR reaction mixture contained all reactions running in duplicate and the real time PCR cycling conditions were as follows: initial denaturation at 95°c for 10 min, followed by cycles of denaturation at 95°c for 10s, annealing at 60°c for 20s. In real-time PCR studies, relative quantification or relative gene expression is the parameter used for relative fold changes in expression of target genes (Ku70 and XRCC4), normalized to an internal reference (HPRT gene) and a relevant untreated and unirradiated control. This parameter is calculated according to $2^{-\Delta\Delta CT}$ formula. ΔΔCT is the difference between the mean ΔCT (treatment group) and mean ΔCT (control group) and ΔCT is the difference between the mean CT gene of interest and the mean CT of internal control gene in each sample. CT is the threshold cycle, i.e. the cycle number at which the

PCR product crosses the threshold of detection. For each group at 8, 24 and 48 h post-irradiation, six independent blood samples were assessed. Assays were performed in duplicate for each sample.

STATISTICAL ANALYSIS

For analyzing data the mean ± SEM of experiments per group were presented and one-way analysis of variance (ANOVA) was performed in order to compare the differences among groups, followed by Tukey's test for multiple comparison. The significance level was set at 0.05.

RESULTS

The expression change of Ku70 and XRCC4 genes in the rat peripheral blood at 8, 24 and 48 h after irradiation of sublethal dose of 2Gy whole body radiation were analyzed by real-time quantitative PCR. The results have been summarized in Table 1.

The obtained results show that in comparison with the control group, the mRNA levels of Ku70 and XRCC4 genes (normalized against HPRT) have significant change in some groups. Gene expression increased significantly in IR and VEH + IR groups only at 24 h after irradiation compared to control group or both genes. In IR group these changes were 5.9 ($P<0.01$) and 7.67 fold ($P<0.05$) for Ku70 and XRCC4, respectively; and in VEH + IR group the fold changes were 5.39 ($P<0.01$) and 7.01 ($P<0.05$) for Ku70 and XRCC4, respectively. Expression of these two genes also increased significantly in MEL+IR group at all three times after irradiation in comparison with control group (Fig. 1). Expression changes for XRCC4 gene at 8, 24 and 48 h were 14.42, 41.93, and 5.39 fold respectively ($P<0.01$), whiles these results for Ku70 gene were 7.36, 29.65, and 4.47 fold, respectively ($P<0.01$). We found no significant differences in the gene expression of Ku70 and XRCC4 in other groups compared to control group.

Results showed that regulation changes of Ku70 and XRCC4 genes were significantly different in MEL + IR group at three times after irradiation compared to IR group as well. We calculated a ratio (fraction) in order to understand the effect of melatonin on the NHEJ repair pathway in the current study. Thus relative expression of MEL+ IR group was divided by relative expression of IR group. This ratio is going to show the role of melatonin in regulating Ku70 and XRCC4 genes. Based on the results for Ku70 and XRCC4 genes, it can be said that melatonin upregulates Ku70 gene as 6.94, 5.02 and 5.02 fold higher and XRCC4 gene as 5.55, 5.47 and 6.91 fold higher at 8, 24 and 48 post- irradiation, respectively (Fig.2). It can be seen that melatonin upregulates Ku70 more than XRCC4 gene at 8 h and upregulates XRCC4 gene more than Ku70 gene at 48 h after irradiation. In other words, with the increase of post- irradiation time, melatonin decreases gene expression for Ku70 and increases it for XRCC4. Results indicate that melatonin not only prevents cell death by apoptosis but also can repair damaged cells in NHEJ pathway.

DISCUSSION

Some studies have been conducted on the protective role of melatonin against oxidative DNA damage and the effects of melatonin on cell cycle and apoptosis. Before conducting our study, it was very important to know if damaged cells which must be killed have been previously repaired or not. The effect of melatonin on apoptosis has been studied previously by Mohseni et al. (2012), Shirazi et al. (2010), and Rezaeejam et al. (2015) whose results showed that melatonin decreases relative expression of pro-apoptotic BAX and increases anti-apoptotic Bcl-2 genes. In this basis, current study

Table 1: Real-time quantitative RT-PCR analysis of the fold change of Ku70 and XRCC4 at various time points after irradiation relative to control group.

Group	8 h		24 h		48 h	
	Ku70	XRCC4	Ku70	XRCC4	Ku70	XRCC4
CON	1	1	1	1	1	1
VEH	0.97	0.98	0.93	0.93	0.93	0.95
MEL	1.02	1.07	1.03	1.07	1.04	1.10
IR	1.06	2.60	5.90	7.67	0.89	0.78
VEH + IR	0.97	2.46	5.39	7.01	0.95	0.75
MEL + IR	7.36	14.42	29.65	41.93	4.47	5.39

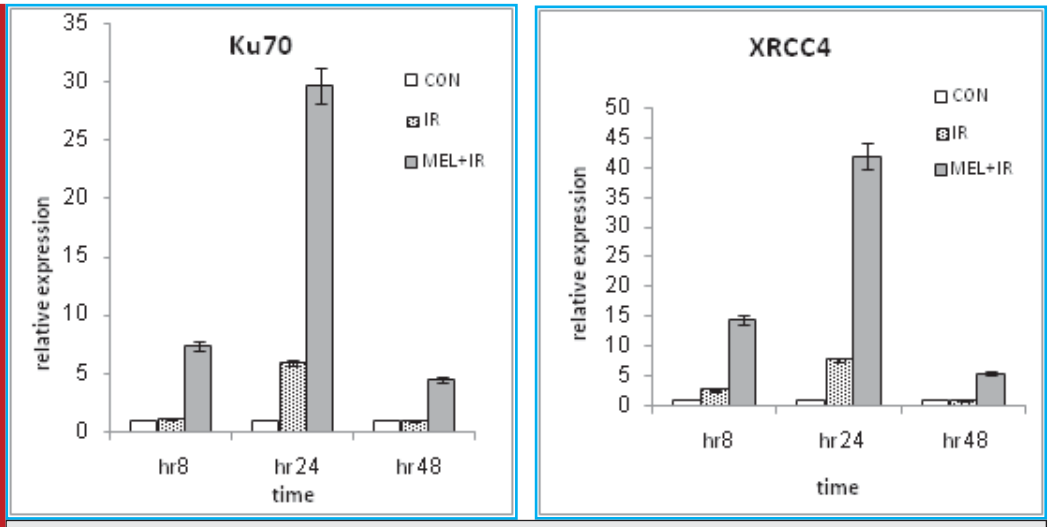

FIGURE 1. Temporal response of Ku70 and XRCC4 genes expression at untreated and pretreated groups. Points denote the mean of responses in six different rats.

investigated the influence of melatonin on DNA DSBs repair-related important genes because of sublethal dose of ionizing radiation in the NHEJ pathway in peripheral blood rat. According to many previous studies, XRCC4, XRCC5 and XRCC6 (KU70) genes are the core of DNA DSBs repair-related genes involved in NHEJ pathway. In this study, the gene expression of Ku70 and XRCC4 were examined using real-time quantitative PCR.

We used 2Gy of 6MV irradiation because it is a standard fraction that is administrated to patients daily in a fractionation regimen and the highest sensitivity for human DNA is obtained for this dose of radiation. The selection of one hour interval between melatonin injection and exposure to gamma radiation was largely based on previous studies (Vijayalaxmi et al. 1999; Hussein et al. 2005). The melatonin concentration was selected based on the experience from the performed studies by other researchers (e.g. Yurtcu et al. 2007) and our previous studies (Mohseni et al. 2012; Shirazi et al. 2010; Rezaeejam et al. 2015) where it was found out this concentration doesn't have any toxicity.

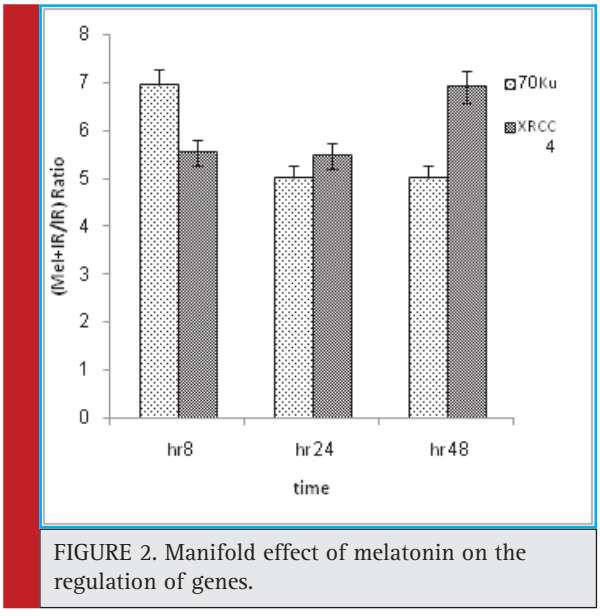

FIGURE 2. Manifold effect of melatonin on the regulation of genes.

Based on the results in this study, since VEH and MEL groups showed no significant difference with control group, we found out that ethanol and melatonin do not have any effects on the expression change of Ku70 and XRCC4 genes alone. On the other hand, IR group had no significant difference with VEH + IR group, so we can say that the injection of ethanol before irradiation doesn't affect the regulation of Ku70 and XRCC4 genes. Furthermore, results illustrated that DBS damages was repaired at 24 h and there was no repair at 8 and 48 h after irradiation in the NHEJ pathway, while the DSBs was repaired at 8, 24 and 48 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.

CONCLUSION

In this study we concluded that melatonin injection before ionizing radiation can increase the expression levels of Ku70 and XRCC4 genes, and melatonin has positive effect on repair in NHEJ pathway. Further inestigation is recommended on the other doses, post-irradiation times and genes related to HR and NHEJ pathways.

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