

Original Article

Protective effects of melatonin agonist on radiotherapy-induced ovarian damage in rats

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Abstract: Objective: To investigate whether melatonin agonist has protective effects on radiotherapy-induced ovarian damage in rats. Methods: Twenty-four SD rats were divided into 4 groups, receiving normal saline (n=6), 200 cGy radiation + normal saline (n=6), 200 cGy radiation + MT (50 mg/kg) (n=6) and 200 cGy radiation + MT (100 mg/kg) (n=6), respectively. All rats were decapitated at two weeks after radiotherapy. Hydroxyl free radical (\bullet OH) levels in blood were detected as the indicator of free radical damage. Levels of serum follicle-stimulating hormone (FSH), estradiol (E2) and the number of follicles were measured as the indicators of ovarian reserve, mitochondrial membrane potential ($\Delta\Psi$), cytochrome C (cyt c) levels and caspase3 activity were assayed as indicators of mitochondrial pathway. Results: Radiotherapy caused a significant increase in the levels of \bullet OH, FSH, cyt c and the activity of caspase3, meanwhile the level of E2, the number of follicles and $\Delta\Psi$ decrease in the Rad group ($P < 0.05$); however in Rad + MT group, these changes were abrogated ($P < 0.05$). Conclusion: melatonin can diminish radiotherapy-induced ovarian damage in female rats, it may be related to the mechanism that MT inhibit mitochondrial pathway induced by radiation.

Keywords: Melatonin, radiotherapy, ovary, rats

Introduction

With the radiation technology advancing, many malignant tumors and autoimmune diseases have been improved, however the damage to ovarian function caused by radiation result in menstrual disorder, amenorrhea, menopausal symptoms, and infertility. How to protect the ovarian function of patients who received systemic radiotherapy or pelvic radiotherapy has become the focus of current research.

Radiation interacts with water to generate some radicals, such as the hydrogen-free radicals and \bullet OH which excessively react with biological molecules. Studies show that the \bullet OH generated by radiation produces 60%-70% of the damage to tissue and DNA [1].

Melatonin, as powerful free-radical scavengers, is capable of passing through all morphophysiological barriers to eliminate \bullet OH in different models of oxidative stress [2, 3]. Furthermore, melatonin can resist radiation by up-regulat-

ing the activity and expression of antioxidant enzymes, including the superoxide dismutases (SOD), catalase, glutathione reductase and glucose-6 phosphate dehydrogenase [4]. Melatonin also possesses the antiapoptotic effect [5], which seems to be concerned with its antioxidant and free radical scavenging action [6]. Furthermore, recent study showed that melatonin exerts the antiapoptotic effects by maintaining the mitochondrial homeostasis and combating mitochondrial oxidative stress [7].

Melatonin, as a direct free radical scavenger and indirect antioxidant, owns high clinical value. Therefore, the present experiment was carried out to investigate the effect of melatonin agonist on radiotherapy-induced ovarian damage in female rats.

Materials and methods

Rats and reagents

24 SD rats, weighting 180-200 g body, were obtained from the Laboratory Animals Depart-

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Table 1. E2 and FSH concentration of 4 groups rats serum (mean \pm SEM)

Experimental group	E2 (pmol/l)	FSH (ng/ml)
Control	6.68 \pm 0.48	0.340 \pm 0.011
Rad	2.83 \pm 0.51*	0.604 \pm 0.028*
Rad + MT (50 mg/kg)	4.26 \pm 0.44*#	0.479 \pm 0.023*#
Rad + MT (100 mg/kg)	5.73 \pm 1.36*#■	0.431 \pm 0.053*#

Compared with the control group, * $P < 0.05$; Compared with Rad group, # $P < 0.05$; and compared with Rad + MT (50 mg/kg), ■($P < 0.05$).

ment of Nanjing Jin Ling hospital, which were raised in a 20-25°C controlled animal care room with 12-hour-sequential periods of light and dark. The animals had free access to normal rat meal and water during the experiments. Vaginal secretion smears of rats were observed at 8:00 every morning; rats with normal estrus cycle were selected to enter the experiment. Melatonin was purchased from the United States Sigma reagent company. ELISA kits which measures rat's follicle stimulating hormone (FSH), estradiol (E2) and cyt c were purchased from AMEKO Reagent Company. Hydroxyl free radical assay kit, Tissue Mitochondria Isolation Kit, Mitochondrial membrane potential assay kit with JC-1 and Caspase3 activity assay kit were purchased from Bi Yun Tian Technology Company.

Administration of melatonin and radiation

The animals were divided into 4 experimental groups: control group, Rad group, Rad + MT (50 mg/kg) group and Rad + MT (100 mg/kg) group. The Control group was i.p. injected with 5 ml alcohol saline (9:1. v/v; 0.9% NaCl and 100% ethanol), Rad group was i.p. injected with 5 ml alcohol saline 2 h before radiation. Rad + MT (50 mg/kg) group, Rad + MT (100 mg/kg) group were i.p. injected with corresponding dose melatonin dissolved in 5 ml alcohol saline 2 h before radiation. Radiotherapy was applied in radiotherapy department of Nanjing Jin Ling hospital, using Swedish Elekta Precise medical linear accelerator. Rad group, Rad + MT (50 mg/kg) group, Rad + MT (100 mg/kg) group received total 200 cGy radiation, that were delivered at a dose rate of 50 cGy/min. During radiation rats were kept in a 30×30 cm sized cage that was subdivided into 6 compartments [8]. All rats were sacrificed during diestrus 2 weeks after radiation.

Determination of hormone

Blood samples were collected from the aortic arch, rats follicle stimulating hormone (FSH) and estradiol (E2) were measured by ELISA kit.

Histological observation

The left ovaries were fixed in 4% paraformaldehyde and embedded in paraffin. Using ultramicrotome, sections were prepared by 1 mm in thickness and stained by HE, the largest cross sections of ovary were observed in this study, primordial follicle, primary follicle, secondary follicles and mature follicle were counted under a light microscope [9].

Determination of hydroxyl free radical

Fenton reaction is the chemical reaction of hydroxyl free radical, the amount of hydrogen peroxide is proportional to the amount of hydroxyl produced by Fenton reaction, when adding electron acceptor, Griess reagent will form red color material, the color of which is directly proportional to the amount of hydroxyl groups.

Determination of mitochondrial membrane potential

Using Tissue Mitochondria Isolation Kit to isolate cytosolic and mitochondria of the left ovarian tissue, JC-1 is an ideal fluorescent probe widely used for the detection of mitochondrial membrane potential $\Delta\Psi$ M, which can detect the mitochondrial membrane potential of cell, tissue or purified mitochondria. When the mitochondrial membrane potential is high, JC-1 gather in the mitochondrial matrix to form polymer (J-aggregates), producing a red fluorescence; when the mitochondrial membrane potential is low, JC-1 cannot gather in the matrix of mitochondria, the monomer (JC-1 monomer) can produce a green fluorescent. The relative proportion of red and green fluorescence is commonly used to measure mitochondrial depolarization ratio, according to kit instructions.

Determination of cytochrome C

Use Tissue Mitochondria Isolation Kit to isolate cytosolic and mitochondria of the left ovarian tissue. Cytochrome C is measured by ELISA kit.

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Table 2. Follicle number of 4 groups rats (mean \pm SEM)

Follicle stage	Primordial	Primary	Secondary	Mature	Total
Experimental group					
Control	3.00 \pm 0.89	10.50 \pm 1.05	5.00 \pm 0.89	3.17 \pm 0.75	21.67 \pm 1.97
Rad	1.33 \pm 0.82	5.33 \pm 1.03	2.50 \pm 1.05	2.33 \pm 0.52	11.50 \pm 2.43*
Rad + MT (50 mg/kg)	3.33 \pm 0.52	7.00 \pm 0.89	3.50 \pm 0.55	1.67 \pm 1.21	15.50 \pm 1.05*#
Rad + MT (100 mg/kg)	2.83 \pm 0.98	8.33 \pm 1.37	4.50 \pm 1.05	2.33 \pm 1.21	18.00 \pm 2.28*#■

Compared with the control group, * $P < 0.05$; Compared with Rad group, # $P < 0.05$; and compared with Rad + MT (50 mg/kg), ■($P < 0.05$).

Determination of caspase3

The right ovaries were homogenized centrifuged to get the clear cytosol, equal amount of cytosolic protein was used to assay caspase3 activity. Caspase3 catalyze Ac-DEVD-pNA to produce yellow substance pNA, the absorbance of pNA released as a result of caspase3 activity was measured at 405 nm.

Data analysis

All the data were expressed as mean \pm SEM. The comparison among groups applies One Way ANOVA analysis of variance, the comparison between the two groups applies LSD and S-N-K method, $P < 0.05$ was considered as statistically significant.

Results

Estrous cycle of rats

All rats in each group had normal estrous cycle of 4 days before radiation. After radiation, there were only metestruses or diestrus left in Rad group, Rad + MT (50 mg/kg) group, and Rad + MT (100 mg/kg). For Rad + MT (50 mg/kg) group and Rad + MT (100 mg/kg) group, normal estrous cycle recovered one week after radiation. The control group had no changes of estrous cycle.

E2 and FSH levels of blood (Table 1)

E2 level: after irradiation, the level of E2 in Rad group was lower than that in normal control group ($P < 0.05$); the level of E2 in Rad + MT (50 mg/kg) group and Rad + MT (100 mg/kg) group was significantly higher than that in Rad group ($P < 0.05$), and the level of E2 in Rad + MT (100 mg/kg) group was significantly higher than that in Rad + MT (50 mg/kg) group ($P < 0.05$).

FSH level: after irradiation, the level of FSH in Rad group was higher than that in normal control group ($P < 0.05$); the level of FSH in Rad + MT (50 mg/kg) group and Rad + MT (100 mg/kg) group was significantly lower than that in Rad group ($P < 0.05$), and the level of FSH showed no significant difference between Rad + MT (50 mg/kg) group and Rad + MT (100 mg/kg) group ($P > 0.05$).

Number of follicles in the largest cross sections (Table 2; Figure 1)

The number of follicles in Rad group was lower than that in normal control group ($P < 0.05$); The number of follicles in Rad + MT (50 mg/kg) group and Rad + MT (100 mg/kg) group was significantly higher than that in Rad group ($P < 0.05$), and The number of follicles in Rad + MT (100 mg/kg) group was significantly higher than that in Rad + MT (50 mg/kg) group ($P < 0.05$).

Concentrations of hydroxyl free radical, mitochondrial membrane potential, concentrations of cytochrome C and the activity of caspase3 (Tables 3-6)

In accordance with the control group, Rad + MT (100 mg/kg) group, Rad + MT (50 mg/kg) group and Rad group, concentrations of \bullet OH, concentrations of cyt C and the activity of caspase3 increased, and $\Delta\Psi$ decreased ($P < 0.05$).

Discussion

Ovarian damage is a major long-term complications of patients who received radiotherapy, radiotherapy of high dose will damage the ovarian function of all ages, causing amenorrhea and the decline of fertility [10], thus improving the survival rate of patients and protecting ovarian function from radiotherapy damage

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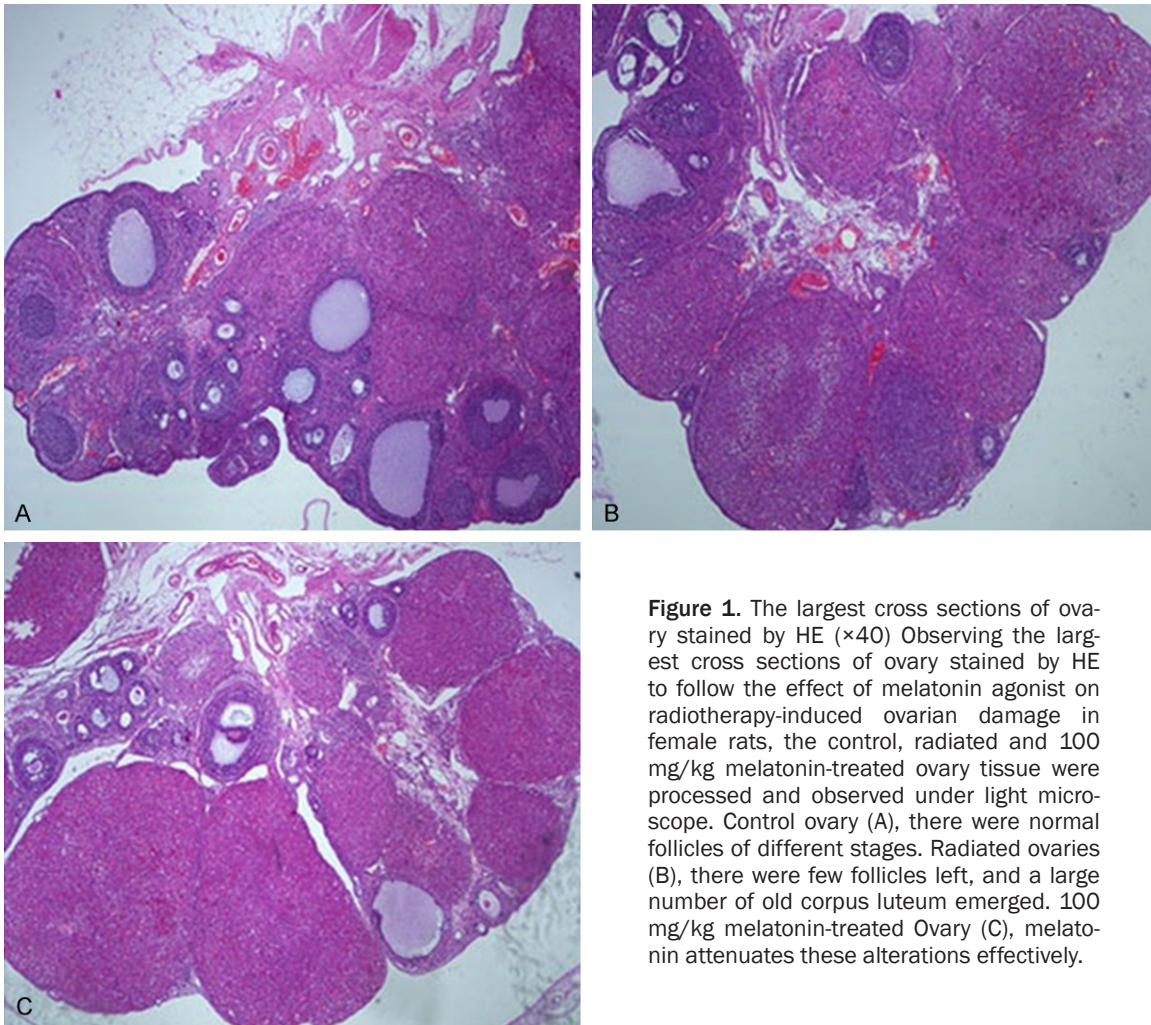


Figure 1. The largest cross sections of ovary stained by HE ($\times 40$) Observing the largest cross sections of ovary stained by HE to follow the effect of melatonin agonist on radiotherapy-induced ovarian damage in female rats, the control, radiated and 100 mg/kg melatonin-treated ovary tissue were processed and observed under light microscope. Control ovary (A), there were normal follicles of different stages. Radiated ovaries (B), there were few follicles left, and a large number of old corpus luteum emerged. 100 mg/kg melatonin-treated Ovary (C), melatonin attenuates these alterations effectively.

have become the focus of current research. Establishing an effective rat ovarian damage model induced by radiotherapy to discuss the mechanism of radiation on ovarian damage and its prevention is of great significance.

Ovary is very sensitive to radiation in the female reproductive systems, with the increase of radiation dose, ovarian function damage is more serious [11]. The researchers usually adopt rat estrous cycle to evaluate the ovarian function. In Rad group, the estrus of rats disappeared, the ovarian tissue morphology was characterized by ovarian atrophy, ovarian cortical thickening, structure disorder, interstitial fibrosis, growing follicles declining and atresia follicles increasing, thus the decline of ovarian function was obvious, coinciding with its change of estrous cycle. Studies have shown that total number of follicles is the important index reflecting ovarian function [12], in this study the

total number of follicles decreased significantly after radiotherapy. Ovarian tissue atrophy, cortical thickening and follicles apoptosis cause the production of E2 decreasing, then the lower E2 levels in the blood will stimulate hypothalamus pituitary gonadal axis to secrete more FSH. Compared with the control group, serum E2 levels declined and FSH levels increased in Rad group. Thus the reduction of estrous cycle, the decline of the serum E2, the increase of serum FSH, atrophy of ovarian tissue and the decrease of follicles number demonstrate the successful build of rats ovarian damage model induced by radiotherapy.

This study showed that exogenous melatonin effectively reduced ovarian damage induced by ionizing radiation. In the rats ovarian damage model induced by radiotherapy, a large number of highly reactive $\cdot\text{OH}$ produced by ionizing radiation led to the oxidative stress. melatonin

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Table 3. Concentrations of •OH in 4 groups rats (mean ± SEM)

Experimental group	Concentrations of •OH (U/ml)
Control	106.8±46.5
Rad	615.3±44.3*
Rad + MT (50 mg/kg)	395.3±42.5*#
Rad + MT (100 mg/kg)	230.1±37.2*#.#

Compared with the control group, * $P < 0.05$; compared with Rad group, # $P < 0.05$; and compared with Rad + MT (50 mg/kg), #($P < 0.05$).

Table 4. Mitochondria membrane potential in 4 groups rats

Experimental group	The ratio of JC-1 polymer and JC-1 monomer fluorescence intensity
Control	1.335±0.179
Rad	0.615±0.074*
Rad + MT (50 mg/kg)	0.785±0.094*#
Rad + MT (100 mg/kg)	1.046±0.134*#.#

Compared with the control group, * $P < 0.05$; compared with Rad group, # $P < 0.05$; and compared with Rad + MT (50 mg/kg), #($P < 0.05$).

Table 5. Cytochrome-C Concentrations in 4 groups rats ($\bar{x} \pm s$)

Experimental group	Concentrations of cyt c (nmol/L)
Control	151.5±7.5
Rad	235.1±7.1*
Rad + MT (50 mg/kg)	201.6±8.5*#
Rad + MT (100 mg/kg)	179.4±8.6*#.#

Compared with the control group, * $P < 0.05$; Compared with Rad group, # $P < 0.05$; and compared with Rad + MT (50 mg/kg), #($P < 0.05$).

Table 6. Capase3 activity of ovarian tissue (mean ± SEM)

Experimental group	pNA release, OD at 405 nm
Control	0.14±0.03
Rad	0.50±0.05*
Rad + MT (50 mg/kg)	0.36±0.05*#
Rad + MT (100 mg/kg)	0.26±0.06*#.#

Compared with the control group, * $P < 0.05$; compared with Rad group, # $P < 0.05$; and compared with Rad + MT (50 mg/kg), #($P < 0.05$).

effectively inhibit the production of •OH to maintain the normal redoxstate of ovarian tissues, especially 100 mg/kg melatonin greatly restrained oxidative stress of ovarian tissue. meanwhile it was found that the mitochondrial apoptotic pathway was also involved in ovarian

tissue damage, melatonin inhibited it and mitigated the ovarian damage via mitochondrial apoptotic pathway.

Mitochondrion is one cystic structure surrounded by double membranes, proton pump transfer large quantities of proton from the matrix of mitochondrial to form $\Delta\Psi$. The fluctuation of $\Delta\Psi$ is associated with many mechanisms. Studies show that the occurrence of apoptosis make $\Delta\Psi$ decline, which results to opening of mitochondrial permeability transition pores (MPTP) [13], then cyt C is released into the cytoplasm, causing caspase cascade. Caspase is a group of protease with similar structure, which is mainly responsible for the selective cleavage of certain proteins, causing target protein activated or deactivated [14]. At present, many studies suggest there are the relationships between Caspase3 and $\Delta\Psi$, the study shows: all animal cells have similar apoptotic mechanisms [15]. Caspase plays a very important role in the apoptosis, which is called a Caspase dependent apoptosis [16, 17].

In this study, melatonin inhibited the decline of $\Delta\Psi$, possibly by preventing the opening of MPTP; the direct control of melatonin on MPTP cannot be ignored. Melatonin possess the antiapoptosis properties due to its antioxidant effects, melatonin limited mitochondrial glutathione loss and protected mitochondrial protein from oxidative stress damage by scavenging oxygen free radicals and nitrogen radicals. In addition, the direct action of melatonin on mitochondrial greatly improved the activity of the electron transport chain, thus reducing the damage of mitochondrial DNA [18].

By analyzing the correlation between ovarian damage, oxidative stress index (•OH) and the mitochondrial pathway index ($\Delta\Psi$, cyt C, Caspase3) were concluded. We demonstrate the obvious activation of oxidative stress and mitochondrial pathway in the Rad group rats, thus it was speculated that ionizing radiation caused the ovarian damage by oxidative stress, and the injury occurs via the mitochondrial pathway. Comparing serum E2, FSH as well as the total number of follicles, it was found that melatonin can obviously reduce ovarian damage induced by ionizing radiation, in addition, melatonin inhibit the production of •OH, the decrease of $\Delta\Psi$, the release of cyt C

and the activation of caspase3. Thus, it can be concluded that melatonin has strong antioxidant and antiapoptotic properties which is expected to be applied to clinical treatment to ovarian damage of patients receiving radiotherapy.

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Disclosure of conflict of interest

None.

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