Journal of Reproduction and Development, Vol. 66, No 1, 2020

—Original Article—

Melatonin protects against Epirubicin-induced ovarian damage

Naiqiang WANG[1)*], Hua LI[2)*], Yunqing ZHU[1)], Na LI[1)], Zi-Jiang CHEN[3, 4)] and Cong ZHANG[1, 3, 4)]

[1)]*Key Laboratory of Animal Resistance Research, College of Life Science, Shandong Normal University, Shandong 250014, China*

[2)]*Department of Gynecology and Obstetrics, Ji'nan Maternity and Child Care Hospital, Shandong 250001, China*

[3)]*Center for Reproductive Medicine, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200135, China*

[4)]*Shanghai Key Laboratory for Assisted Reproduction and Reproductive Genetics, Shanghai 200135, China*

Abstract. One major side effect of chemotherapy that young women with cancer suffer from is ovarian damage. Therefore, it is necessary to study the pathogenesis of chemotherapeutic drugs in order to develop pharmaceutical agents to preserve fertility. Epirubicin is one of the commonly used chemotherapy drugs for breast cancer patients. This research explored the side effects of epirubicin in mice. We found that epirubicin significantly reduced the body weight, the weight of the ovaries and uteri, and the pups' number, while melatonin, which is extremely resistant to oxidation, significantly reduced these damages. Moreover, co-treatment with melatonin prevented epirubicin-induced decrease in E_2 and progesterone, and the loss of follicles. Mechanism study showed that melatonin significantly reduced the levels of proapoptotic genes *p53*, *Caspase3*, and *Caspase9* while it upregulated antiapoptotic factors *Bcl-2* and *Bcl2l1*, and antioxidant genes superoxide dismutase 1 and catalase compared with the epirubicin group. In addition, melatonin markedly reduced reactive oxygen species (ROS) and the transcription of *Caspase12* and *Chop*, which is vital in endoplasmic reticulum stress (ERS)-mediated apoptosis. These results indicate melatonin protects against epirubicin-induced ovarian damage by reducing ROS-induced ERS. Therefore, melatonin has a therapeutic potential for the protection of ovarian function and preservation of fertility during chemotherapy.

Key words: Endoplasmic reticulum stress (ERS), Epirubicin, Melatonin, Ovarian damage, Reactive oxygen species (ROS)

(**J. Reprod. Dev. 66:** 19–27, 2020)

Globally, there were approximately 2.1 million newly diagnosed cases of female breast cancer in 2018, accounting for nearly a quarter of female cancer cases (24.2%). It is the most common cancer in 154 out of 185 countries, and is the first cause of cancer deaths (15.0%) in more than 100 countries [1]. Although chemotherapy increases survival and decreases recurrence, it would induce ovarian damage, and result in chemotherapy-caused amenorrhea [2–5]. Therefore, to explore the mechanisms and to identify protective agents against ovarian toxicity during chemotherapy is of high priority.

Epirubicin, the 4'-epimer of doxorubicin, intercalates into DNA double-stranded base pairs, and thus inhibits nucleic acid synthesis, which is most effective for metastatic diseases [6]. It is extensively applied to treat tumors of various types, including lung cancer, stomach cancer, and various types of cancers, especially breast cancer [6–8]. Although epirubicin is very effective for various types of cancers, its clinical application is confined due to its severe side effects, for instance, cardiotoxicity, nephrotoxicity, hepatotoxicity, and reproductive system dysfunction [9–12]. Unfortunately, up to now, a detailed mechanistic study of its side effects is lacking. Though the pathogenesis is complex, epirubicin is currently known in terms of cardiotoxicity due to the production of reactive oxygen species (ROS) [13]. Considering the effectiveness and toxicity of epirubicin, some agents have been applied together with it to overcome these side effects [10–12, 14, 15].

The indoleamine melatonin was first known as an endogenous product of the pineal gland. However, many organs such as the placenta, ovary, and retina have been discovered to produce this important substance [16, 17]. Melatonin has a strong anti-oxidative ability compared with other distinguished antioxidants, including vitamin C, vitamin E, and glutathione [18]. In addition, various investigations have demonstrated that exogenous melatonin can also protect the ovary, uterus, testes, kidney, lung, and nervous system against oxidative stress [19–23]. Furthermore, melatonin has great potential for application because it decreases the side effects of antineoplastic drugs synergistically through eliminating peroxyl radicals, superoxide anion radicals, and hydrogen peroxide [24]. Previous research showed that epirubicin impairs rat cardiac function because of the deformation and degeneration of the mitochondria, formation of vacuole, and dysfunctional myofibrillary structures, whereas the degradation was rescued by treatment with melatonin [25]. However, the roles of epirubicin and melatonin in mouse reproduction are unknown.

We hypothesized that melatonin can be used to prevent epirubicin-

Received: July 15, 2019
Accepted: October 21, 2019
Advanced Epub: November 15, 2019

Correspondence: C Zhang (e-mail: zhangxinyunlife@163.com)
* N Wang and H Li contributed equally to this work.

induced ovary damage by removing ROS. Therefore, we performed experiments to clarify the role and the mechanisms of melatonin using mice. Our data indicate that melatonin can significantly reduce epirubicin-induced ovary damage due to its strong antioxidation role.

Materials and Methods

Animals

Female ICR mice (five weeks old) were purchased from Laboratory Animal Core of Shandong University, and maintained under constant temperature (23 ± 2ºC) and photoperiod (12L:12D) with food and water *ad libitum*. Mice were treated according to the Guidelines of the Shandong Normal University for the Care and Use of Laboratory Animals [26].

Female ICR mice (six weeks old) were assigned to six treatments randomly, NC (no treatment), Ctrl [vehicle for melatonin (MLT) + vehicle for epirubicin (EPI)], EPI (vehicle for MLT + 3 mg/kg EPI), MLT & EPI (15 mg/kg MLT + 3 mg/kg EPI), MLT+&EPI (30 mg/kg MLT + 3 mg/kg EPI), MLT++&EPI (60 mg/kg MLT + 3 mg/kg EPI). Epirubicin was injected half an hour after melatonin injection and the dosage was determined by referring to the dose of doxorubicin, an isomer of epirubicin used for the mice [27]. All mice received intraperitoneal injection daily at 1800 h for 20 days and the mice were weighed before injection. The mice were sacrificed, and the materials including ovaries and serum were collected on the morning of the 21st day (12 h after the last injection of melatonin and epirubicin) for subsequent experiments. For fertility assay, one reproductively proven male was continually housed with two females for eight months; these females were carefully observed daily until pups were born and the dates were recorded, and the number of pups increased [28].

Serum progesterone and estrogen assay

For hormone level measurement, the mice were selected at estrus according to vaginal smear [29, 30]. Estrogen level in serum was determined using the Estradiol Radioimmunoassay Kit (Jiuding Biological Engineering, Tianjin, China) according to the instructions of the manufacturers. The coefficients of variation (CVs) of intra- and inter-assay were 7.7% and 8.9%, respectively. The lowest concentration that could be detected was 2.1 pg/ml. Progesterone level was determined with Progesterone Radioimmunoassay Kit (Jiuding Biological Engineering). The CVs of intra- and inter-assay were 7.2% and 8.9%, respectively. The lowest concentration that could be detected was 0.03 ng/ml [29, 31].

Follicle count

Ovaries embedded in O.C.T compound (Sakura Finetek, Torrance, CA, USA) were cut at 8 μm and stained with hematoxylin and eosin. Every fifth section was counted, and the number of primordial, primary, preantral, and antral follicles was classified and counted in accordance with a previous study [32]. Briefly, primordial follicle was classified if it had an oocyte surrounded by a single layer of squamous granulosa cells (GCs). A follicle was considered as primary follicle if it had an oocyte surrounded by a single layer of cuboidal GCs, and if there were two or more layers of cuboidal GCs without visible antrum, it was secondary follicle. A follicle was enumerated as preantral follicle when it had one or several small antral space. A follicle was categorized as antral follicle if it had an oocyte surrounded by more than one layer of GCs and a large antral space with follicular fluid. Preovulatory follicle was the largest follicle containing a cumulous GC layer [23, 33]. To avoid counting the same follicle twice, only follicles with a nucleolus in the nucleus were counted. The total number of follicles in one ovary were estimated by multiplying the follicles in the selected samples by 5 [32].

RNA extraction and quantitative PCR

RNA was extracted after homogenization of the ovaries in TRIzol Reagent (Tiangen Biotech, Beijing, China) in accordance with the instructions of the manufacturers. Reverse transcription was conducted using total RNA pretreated with RNase-free DNase (Tiangen Biotech) and Fast Quant RT Kit (Tiangen Biotech). Quantitative PCR was performed using SYBR green master mix in LightCycler R 96 System (Roche Diagnostics, Basel, Switzerland). The primers for real-time PCR are shown in Table 1.

PCR operating conditions were as follows: 3 min at 94ºC; 40 cycles of 10 sec at 94ºC, 15 sec at 60ºC, and 15 sec at 72ºC; 5 min at 72ºC. PCR used endogenous *Actb* as relative reference, and it was repeated at least three times independently. The data were calculated in accordance with a calibrator sample using ΔΔCt method [31, 34, 35].

Western blot analysis

Proteins in ovaries were extracted using RIPA buffer (Beyotime Biotechnology, Haimen, China) and protease inhibitors (Sigma Aldrich, St. Louis, MO, USA), 30 μg protein was electrophoresed. The gel was electrotransferred to a polyvinylidene fluoride membrane (Merck Millipore, Darmstadt, Germany), blocked with 5% nonfat milk for 90 min, and the membrane was then incubated with primary antibodies (CHOP, 1:500 dilution, 60303-1-1g, Proteintech; β-actin, 1:5000 dilution, SC-47778; Santa Cruz Biotechnology) overnight at 4ºC. Subsequently, secondary antibody conjugated with horseradish peroxidase (1:5000; ZB-2305; ZSGB-BIO) was used to detect the primary antibody that had bound to the membrane. The signals were obtained and analyzed using the chemiluminescence kit (Millipore, MA, USA) and Quantiscan software (Biosoft, Cambridge, UK) [26, 36, 37].

Immunohistochemistry

Cryosections (8 μm) were fixed in acetone (–20ºC) for 10 min, followed by washing with PBS,the sections were incubated for 30 min in 0.3% Triton X-100. The samples were then immersed into 0.3% (v/v) hydrogen peroxide in methanol for 20 min. The sections were incubated with 1% (w/v) bovine serum albumin (BSA, Sigma-Aldrich, Santa Clara, CA, USA) for 30 min at 25–35ºC. Then they were incubated with CHOP polyclonal antibody (1:200; 15204-1-AP; Proteintech) diluted in 1% BSA overnight at 4ºC, subsequently with horseradish peroxidase labeled secondary antibody (1:1000; SC-2040, Santa Cruz) diluted in 1% BSA for 1 h at room temperature. The sections were developed with 3, 3'-diaminobenzidine (Vector Labs, CA, USA) and counterstained with hematoxylin. Finally, the sections were counterstained and digitally photographed using an automatic digital slide scanner (Pannoramic MIDI II, 3Dhistech). For negative controls, the primary antibody was replaced with pre-immune serum [38–40].

ROS determination

Freshly made cryosections were incubated with 2',7'-dichlorohydrofluorescein diacetate (Beyotime Biotechnology, Haimen, China) at 37ºC for 20 min. After washing with PBS for three times, the sections were observed at 460 nm with a confocal laser scanning microscope to measure green fluorescence (Leica TCS SPE; Ernst-Leitz-Strasse, Wetzlar, Germany). Fluorescence intensity analysis was conducted with the Image J (Bethesda, MD, USA) [41].

Statistical analysis

Statistical analysis was performed using SPSS 22.0 (IBM, Chicago, IL). Each experiment was repeated at least three times. One-way analysis of variance was applied to analyze the statistical differences between groups, and the least significant difference test was subsequently used. Values were considered statistically significant if $P < 0.05$. The data are expressed as mean ± SEM.

Table 1. Target transcripts and primer sequences used for the quantification of mRNA levels in ovaries

Primer	Sequence	Size (bp)
p53	TACAAGAAGTCACAGCACAT CCAGATACTCGGGATACAAAT	133
Caspase3	CTGACTGGAAAGCCGAAACTC CGACCCGTCCTTTGAATTTCT	189
Caspase9	ACATCCTTGTGTCCTACTCCA TGCTCCAGAATGCCATCCAA	100
Bax	ACTGCCTTGGACTGTGTCTT CATTCCCACCCCTCCCAATA	112
Bcl-2	ACCTGTGGTCCATCTGACCCTC CCAGTTCACCCCATCCCTGA	163
Bcl2l1	TATTGGTGAGTCGGATTGCA GAGATCCACAAAAGTGTCCCA	101
Caspase12	TCTAATGATGAGGATGATGGA GTGTATCTTGGACTTCTGAG	172
Chop	AAGCCTGGTATGAGGATCTGC TTCCTGGGGATGAGATATAGGTG	75
Stim1	GCCAAGGCTAGCAGTAACC AGTCAAGAGAGGAGGCCCAA	117
Orai1	TCAACGAGCACTCGATGCAG TCGCTACCATGGCGAAGC	127
Sod1	AACCAGTTGTGTTGTCAGGAC CCACCATGTTTCTTAGAGTGAGG	139
Sod2	TCAAGAACTTACAGTCACAAGAGG GCACAGGAACACAGTCAGAA	102
Cat	TACACTGAAGATGGTAACTG TTCTCTTCTGGCTATGGATA	103
Gsr	CATGATTCCAGATGTTGACT AATTCGTCTACTAGGATGTG	123
Gpx1	AATCAGTTCGGACACCAGAA CACCATTCACTTCGCACTTC	102
ACTB	GGCTGTATTCCCCTCCATCG CCAGTTGGTAACAATGCCATGT	153

Results

Choosing the best melatonin dose for mice

In order to study whether melatonin has a protective effect on the damage caused by epirubicin and what dose of melatonin can prevent it, three doses of melatonin were administered for 20 days. The results showed that epirubicin alone decreased body weight, ovary weight, and uterus weight significantly, but this decrease was rescued significantly after administration of 60 mg/kg melatonin before epirubicin (Fig. 1A, B, C). Therefore, we chose 60 mg/kg melatonin for the subsequent study of melatonin protection. Furthermore, fertility assay indicated that this dose of melatonin significantly ameliorated pup numbers reduced by epirubicin; nevertheless, the body weight of the pups had no significant change (Fig. 1D, E).

Melatonin restores E_2 and progesterone levels disrupted by epirubicin

To study the roles of epirubicin and melatonin in ovarian function, we examined serum E_2 and progesterone levels. The results showed that exposure to epirubicin decreased serum E_2 (5.07 ± 0.45 *vs*. 9.22 ± 0.86; $P < 0.01$) and progesterone (1.47 ± 0.05 *vs*. 3.71 ± 0.39; P

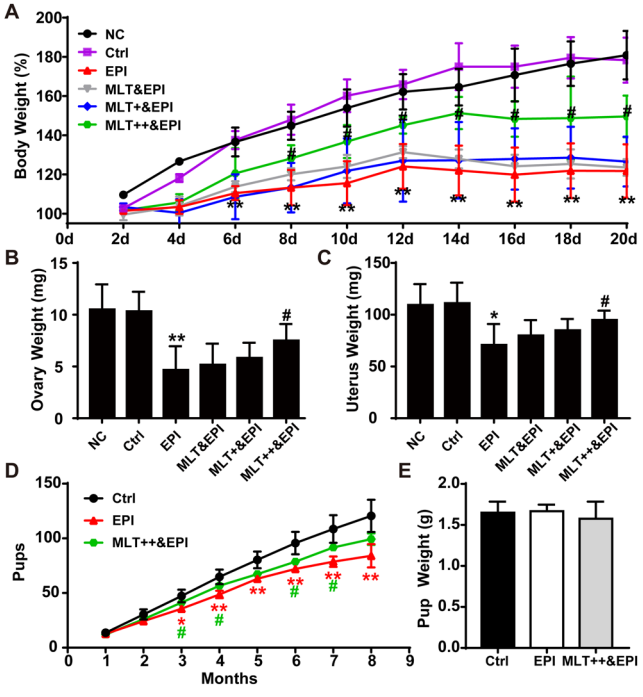

Fig. 1. The body, ovary, and uterus weights and reproductive capacity of the mice. Epirubicin (EPI) with or without melatonin (MLT) were injected into ICR mice for 20 days. (A) The mice were weighed before daily injection. The data from the graph were analyzed using the rate of weight change. n = 24. (B, C) Ovary and uterus weights were measured after sacrifice on the 21st day. n = 8. (D, E) The number and weight of the pups were recorded continuously for 8 months. n = 8. The data are shown as mean values ± SEM. * Significant differences relative to Control (Ctrl) (* $P < 0.05$, ** $P < 0.01$); # Significant differences relative to EPI group (# $P < 0.05$).

< 0.001) levels significantly compared with those in the controls. Upon melatonin co-treatment, the levels of serum E_2 (7.14 ± 0.76 *vs*. 5.07 ± 0.45; P < 0.05) and progesterone (2.44 ± 0.30 *vs*. 1.47 ± 0.05; P < 0.05) were significantly restored compared to those in the epirubicin alone group (Fig. 2).

Melatonin suppresses epirubicin induced follicle loss

Quantification of the follicles at different stages revealed that the number of primordial (340 ± 49 *vs*. 645 ± 78; P < 0.01), secondary (27.5 ± 3.6 *vs*. 64.0 ± 10; P < 0.01), and total (502 ± 67 *vs*. 929 ± 93; P < 0.01) follicles was significantly lower in the epirubicin alone group than those in the controls, whereas treatment together with melatonin ameliorated the number of primordial (473 ± 43 *vs*. 340 ± 49; P < 0.05), secondary (38.9 ± 4.5 *vs*. 27.53 ± 3.6; P < 0.05), and total (664 ± 73 *vs*. 502 ± 67; P < 0.05) follicles significantly (Fig. 3A, C, G). Further, epirubicin alone decreased primary follicles significantly (117 ± 20 *vs*. 199 ± 34; P < 0.05); however, melatonin treatment did not recover the primary follicle loss (133 ± 36 *vs*. 117 ± 20; P > 0.05) (Fig. 3B). Moreover, the number of preantral, antral, and preovulatory follicles was not different following epirubicin and/or melatonin treatment (Fig. 3D, E, F). In brief, melatonin protected follicles against epirubicin-induced ovarian damage by protecting primordial and secondary follicles from loss.

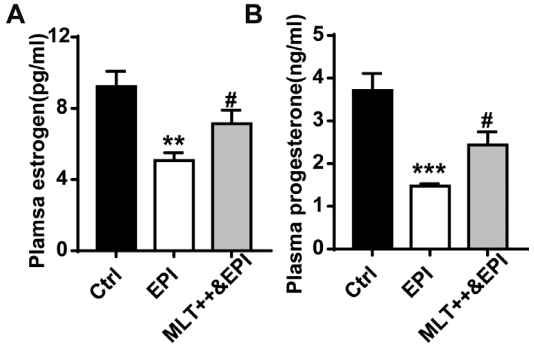

Fig. 2. The serum progesterone and estrogen concentrations of the mice. (A) Serum estrogen and (B) progesterone concentrations of the mice injected with melatonin (MLT) and/or epirubicin (EPI). n = 8. EPI with or without MLT was injected for 20 days, and then the serum was collected on the 21st day in the morning. The data are shown as mean values ± SEM. * Significant differences relative to Control (Ctrl) (** P < 0.01, *** P < 0.001); # Significant differences relative to EPI group (# P < 0.05).

Melatonin suppresses epirubicin-induced apoptosis in ovaries

We then examined the transcription of apoptosis-associated genes in the ovary to investigate the mechanism. The results of real-time PCR indicated that the transcription of proapoptosis genes *p53* (P < 0.01), *Caspase9* (P < 0.001), and *Bax* (P < 0.001) was significantly increased in epirubicin-treated samples compared with those in the controls, whereas the antiapoptotic gene *Bcl-2* (P < 0.05) was downregulated significantly in the epirubicin group. Meanwhile, melatonin co-treatment significantly decreased *p53* (P < 0.001), *Caspase3* (P < 0.001), and *Caspase9* (P < 0.05), and increased *Bcl-2* (P < 0.01) and *Bal2l1* (P < 0.01) compared with those in the epirubicin alone group (Fig. 4). These suggest that epirubicin functions through inducing apoptosis in the ovary, and melatonin can reverse this damage.

Melatonin suppresses epirubicin-induced endoplasmic reticulum stress (ERS) in ovaries

There are many causes and ways of apoptosis. The causes can be divided into physical causes (radiation, temperature, and stimulation) and chemical incentives (active oxygen groups, cytotoxins, inhibitors of DNA and protein synthesis, etc.). Epirubicin is a broad-spectrum anticancer drug, which inhibits nucleic acid synthesis, usually acting on

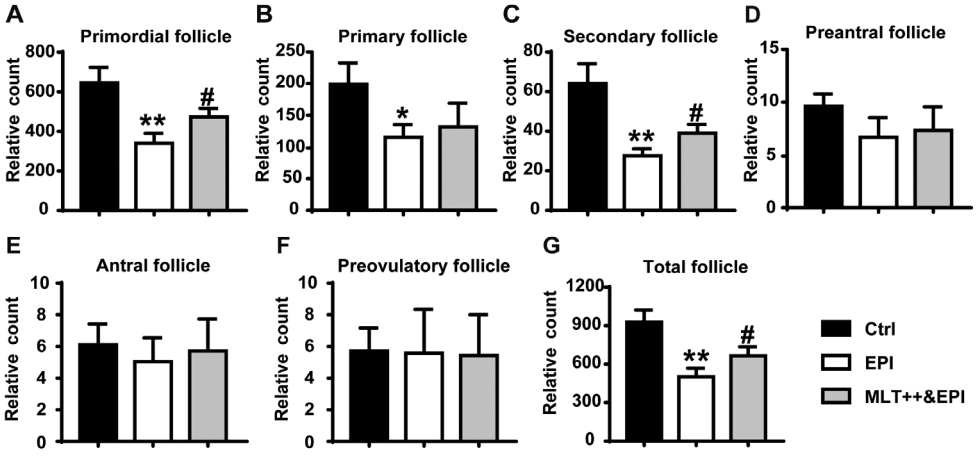

Fig. 3. The relative follicle count of mice. Epirubicin (EPI) with or without melatonin (MLT) were injected into ICR mice for 20 days, and then the ovaries were removed on the 21st day to count the number of (A) primordial, (B) primary, (C) secondary, (D) preantral, (E) antral, (F) preovulatory and (G) total follicles. n = 5. The data are shown as mean values ± SEM. * Significant differences relative to Control (Ctrl) (* P < 0.05, ** P < 0.01); # Significant differences relative to EPI group (# P < 0.05).

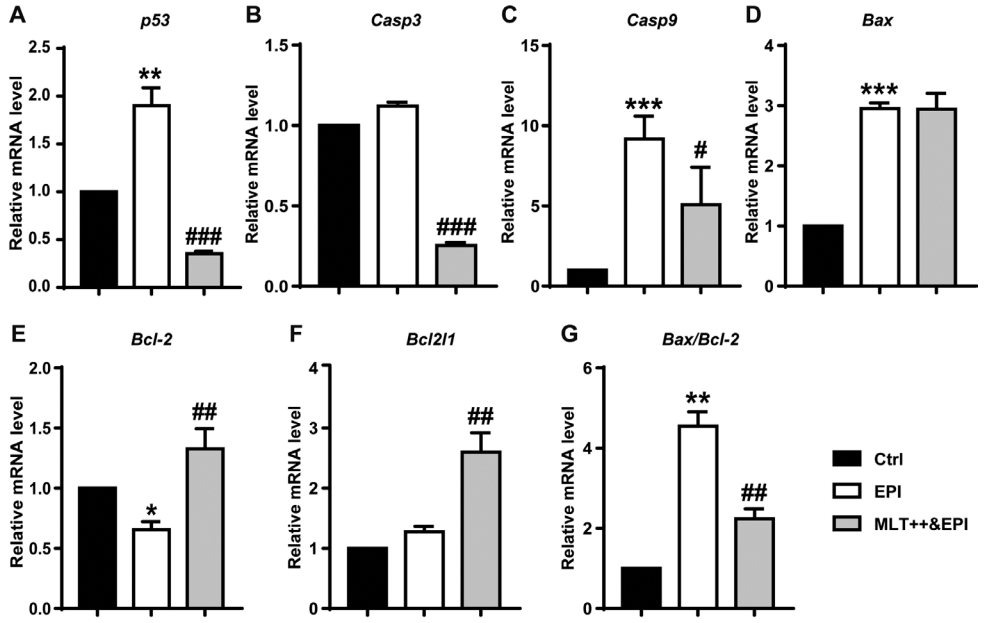

Fig. 4. The expression of the apoptotic-related genes in ovaries. Real-time quantitative PCR analysis of apoptotic-related genes including (A) *p53*, (B) *Caspase3*, (C) *Caspase9*, (D) *Bax*, (E) *Bcl-2*, (F) *Bcl2l1*, (G) *Bax/Bcl2*. Gene expression level was normalized to *ACTB* in the same sample. n = 5. The mice were injected with epirubicin (EPI) and melatonin (MLT) for 20 days, and then the ovaries were collected 12 h after the last injection on the 21st day. The data are shown as mean values ± SEM. * Significant differences relative to Control (Ctrl) (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$); # Significant differences relative to EPI group (# $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$).

cells that are active in nucleic acid synthesis. However, the results of follicle counts in this study showed that epirubicin affected primordial follicles, which are inactive in nucleic acid synthesis. Therefore, we speculated that epirubicin might affect not only the synthesis of nucleic acids, but also protein synthesis, which is an important function of the endoplasmic reticulum. Therefore, we examined ERS related indicators, *Caspase12* and *Chop*. The results of qPCR demonstrated that the expression of *Caspase12* ($P < 0.01$) and *Chop* ($P < 0.01$) was significantly higher in epirubicin treated-samples than that in the control samples, whereas melatonin co-treatment significantly decreased *Caspase12* ($P < 0.01$) and *Chop* ($P < 0.01$) compared with that in the epirubicin alone group (Fig. 5A, B). Moreover, the results of western blot further demonstrated that CHOP ($P < 0.01$) was highly expressed in epirubicin-injured ovaries, and melatonin could decrease CHOP level significantly ($P < 0.01$) (Fig. 5C, D). These results imply that epirubicin induced ERS, which might subsequently cause apoptosis in the ovary.

ERS occurs in follicles at all stages

To further determine the target follicles of epirubicin where ERS occurs, we examined the localization of CHOP in the ovary. The immunohistochemistry results showed that CHOP was much higher in all the follicles treated with epirubicin than those in the controls (Fig. 6B). Moreover, the expression of CHOP decreased in the melatonin co-treatment group (Fig. 6C).

Melatonin decreases ROS induced by epirubicin

To investigate epirubicin-induced ERS in the ovary, we examined

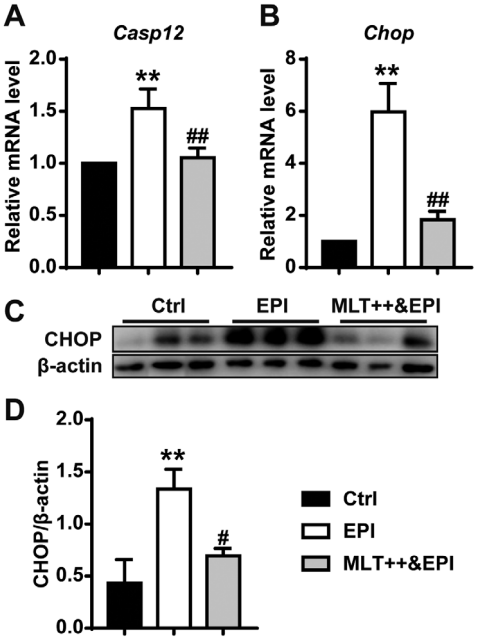

Fig. 5. The expression of *Casp12* and *Chop* in ovaries. (A, B) Expression of *Caspase12* and *Chop* mRNA was determined using real-time PCR. (C, D) The protein level of CHOP was analyzed by Western blot. n = 5. The mice were injected with epirubicin (EPI) and melatonin (MLT) for 20 days first, then the ovaries were taken on the 21st day 12 h after the last injection. The data are expressed as mean ± SEM. * Significant differences relative to Control (Ctrl) (** $P < 0.01$); # Significant differences relative to EPI group (# $P < 0.05$, ## $P < 0.01$).

Ca^{2+} sensor and oxidative related genes. Real-time PCR results indicated that the levels of Ca^{2+} sensor related genes; *Stim*1 and *Orai*1 did not change significantly (Fig. 7A, B). While the mRNA levels of oxidative-related genes, superoxide dismutase (*Sod*1) ($P < 0.001$), *Sod*2 ($P < 0.01$), and catalase (*Cat*) ($P < 0.001$) were significantly lower in the epirubicin group than those in the control, whereas melatonin co-treatment significantly increased *Sod*1 ($P < 0.05$) and *Cat* ($P < 0.01$) compared with those in the epirubicin alone group (Fig. 7C, D, E). Meanwhile, melatonin decreased the expression of oxidative-related gene glutathione reductase (*Gsr*), which was highly expressed in the ovary treated with epirubicin alone (Fig. 7F). However, the expression of another oxidative-related gene glutathione peroxidase (*Gpx*1) was not significantly changed (Fig. 7G). In addition, subsequent experiments showed that ROS level in the ovary treated with melatonin reduced significantly compared with that in the epirubicin alone group ($P < 0.05$) (Fig. 8). These results implied that ERS caused by epirubicin may be due to the increase in ROS in the ovary caused by epirubicin.

Discussion

Our study demonstrated that the chemotherapy drug epirubicin reduced the body weight, the weight of the ovaries and uteri, and the pups' number significantly, while melatonin markedly repaired these damages. In addition, co-treatment with melatonin restored the levels of E_2 and progesterone and the follicle numbers. Mechanism study discovered that melatonin decreased the levels of proapoptotic genes while it upregulated antiapoptotic factors and antioxidant genes significantly. Moreover, melatonin markedly reduced ROS- and ERS-mediated apoptosis, and thus, prevented apoptosis caused by chemotherapy.

There are many follicles of different stages in the mammalian ovaries. Every follicle has an oocyte accompanied with supporting GCs. The ovarian follicle pool is determined early in life and the depletion of the follicles leads to reproductive senescence [42]. A previous study has demonstrated that chemotherapy induced follicle apoptosis, resulting in impaired ovarian function, and even sterility [43]. For instance, the chemotherapeutic agent doxorubicin induces follicular apoptosis, affects follicular development, hormone secretion, oocyte maturation, and shortens the fertility window [27, 44, 45]. Therefore, to determine whether epirubicin, an isomer of doxorubicin, behaves similar to doxorubicin in the ovary is of great significance due to its popular application in clinics. Our results showed that epirubicin induced follicle loss through ERS induced by ROS, which is critical for the treatment of young women with cancer. Further investigations demonstrated that epirubicin reduced the levels of serum E_2 and progesterone significantly. These steroids are critical factors for follicular development and for the pituitary secretion of gonadotropin [31, 46]. Abnormal synthesis of E_2 and progesterone might result in a reproductive relevant disorder.

As early as 1997, researchers found that the antitumor drug doxorubicin caused apoptosis in mouse oocytes, while Bax-deficient oocytes were resistive to doxorubicin-induced apoptosis [47]. Other studies also showed that doxorubicin induces apoptosis in H9c2 cardiomyocytes by increasing ROS, which can result in ERS and subsequently initiate apoptosis via CHOP and caspase12 [48, 49]. In

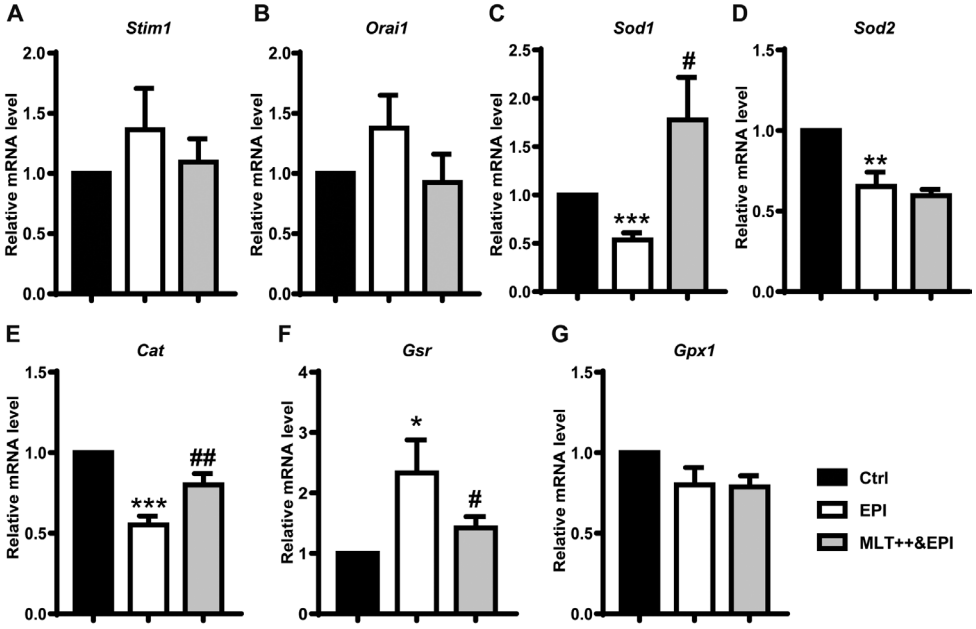

Fig. 7. The expression of *Stim1*, *Orai1*, and oxidative-related genes in the ovaries. Real-time quantitative PCR analysis of Ca^{2+} sensors including (A) *Stim1*, (B) *Orai1* and oxidative-related genes including (C) *Sod1*, (D) *Sod2*, (E) *Cat*, (F) *Gsr*, (G) *Gpx1*. Gene expression level was normalized to *ACTB* in the same sample. n = 5. The mice were administered with epirubicin (EPI) and melatonin (MLT) for 20 days, and the ovaries were collected on the 21st day in the morning. The data are shown as mean values ± SEM. * Significant differences relative to Control (Ctrl) (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$); # Significant differences relative to EPI group ($^{\#} P < 0.05$, $^{\#\#} P < 0.01$).

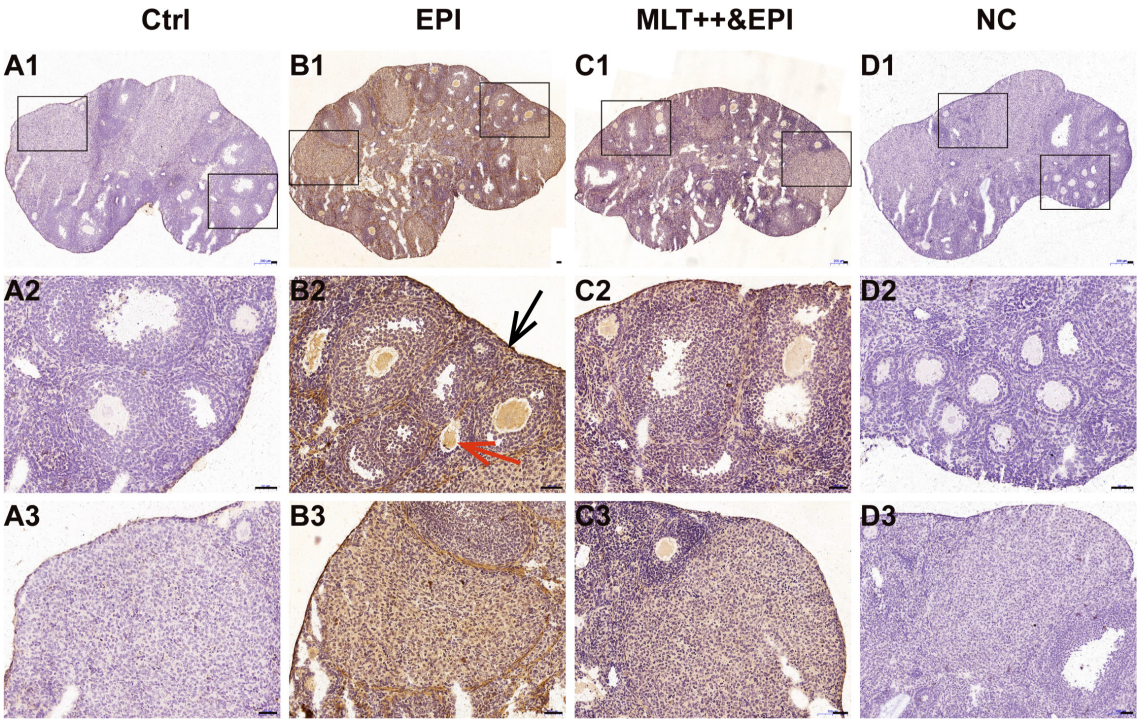

Fig. 6. The localization of CHOP in ovaries. Localization of CHOP in the ovary of (A) control group, (B) epirubicin (EPI) treated group, (C) EPI and melatonin (MLT) co-treatment group, and (D) negative control. n = 5. The mice were administered with EPI and MLT for 20 days previously, and the ovaries were removed on the 21st day. Black arrow shows a primordial follicle, while red arrow indicates a secondary follicle. Scale bars represent 50 μm.

our research, epirubicin also downregulated the antiapoptotic gene (*Bcl-2*), and antioxidant genes (*Sod*1*, Sod*2*,* and *Cat*) significantly, and ultimately increased the ROS in the ovary; meanwhile, epirubicin treatment upregulated proapoptotic genes (*p53*, *Caspase9* and *Bax*), as well as *Caspase12* and *Chop*, which are thought to mediate ERS-specific apoptotic pathways [50, 51]. The main storage site of Ca^{2+} is the endoplasmic reticulum; therefore, the disruption of the accumulation would facilitate ERS [52]. However, the expression of *Stim*1 and *Orai*1, the main regulators of calcium homeostasis, did not change significantly in our study. Therefore, we believe that epirubicin could induce apoptosis in mouse ovary by inducing ERS caused by ROS.

The expression of glutathione reductase was upregulated by epirubicin compared with that in the control. This unexpected increase might be the consequence of a compensatory mechanism. When catalase is insufficient, glutathione reduces H_2O_2 to H_2O under the catalysis of glutathione peroxidase, glutathione itself is oxidized to oxidized glutathione; subsequently, it is reduced to glutathione by glutathione reductase. This step consumes nicotinamide adenine

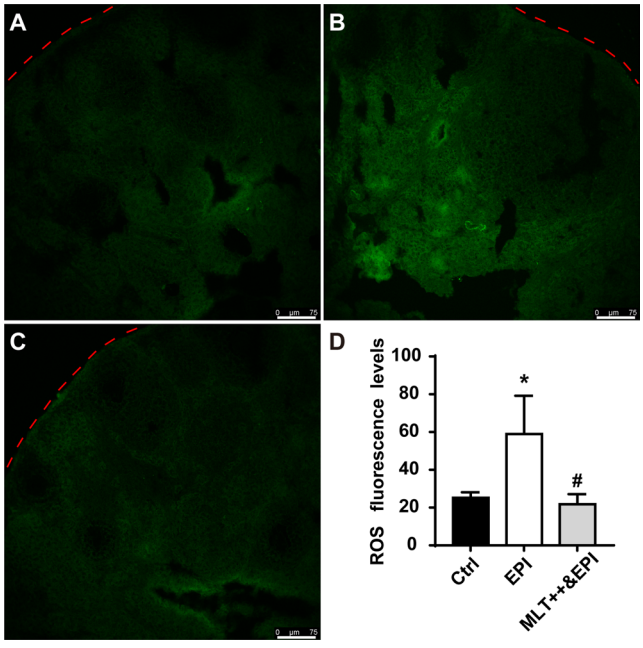

Fig. 8. The effects of epirubicin and melatonin on ROS in ovaries. The ROS fluorescence of the ovary in (A) control group, (B) epirubicin (EPI) treated and (C) melatonin (MLT) co-treatment group. (D) Calculated ROS fluorescence levels in the ovary; Green for ROS. Red broken line shows the borders of ovaries. Scale bars represent 75 μm. n = 4. EPI with or without MLT was injected for 20 days, then the ovaries were obtained the next morning on the 21st day. The data are shown as mean values ± SEM. * Significant differences relative to Control (Ctrl) (* $P < 0.05$); # Significant differences relative to EPI group (# $P < 0.05$).

dinucleotide phosphate (NADPH) [53], which is a hydrogen donor for many anabolic reactions in body, including the synthesis of cholesterol, corticosteroids, and sex hormones. However, further study is necessary to clarify the upregulation of glutathione reductase induced by epirubicin.

Some studies have indicated that chemotherapy induces dormant primordial follicles to go into apoptosis, which results in ovarian failure and sterility. Whereas others show that chemotherapy does not cause primordial follicle apoptosis, but causes the apoptosis of the follicles that grow actively, and promotes primordial follicles to become primary follicles, which results in primordial follicle pool run out [23, 43, 54]. Our results indicated that epirubicin treatment significantly reduced primordial follicles, primary follicles,secondary follicles, and total follicles. These results suggest that epirubicin did not activate primordial follicles; on the contrary, it induced them to undergo apoptosis. Under normal physiological conditions, activated primordial follicles undergo continuous growth and development, but only a few dominant follicles can develop to ovulatory stage; most follicles become atretic [42]. In our study, epirubicin did not reduce the number of preantral, antral, and preovulatory follicles. This suggested that epirubicin did not completely block the promotion of gonadotropin on dominant follicle development. It is also possible that epirubicin achieved some balance between activating follicles and inducing follicle apoptosis, but this hypothesis requires more investigation. In addition, numerous studies have reported that 'small' follicles, including primordial, primary, and secondary follicles, were more susceptible to chemotherapeutic damage, while the development of 'large' follicles were more stable [26, 46–49], which might be the reason why they were more resistant to epirubicin-induced damage in our study. However, epirubicin might also cause potential damage to large follicles and eventually cause fertility decline. Moreover, this study demonstrated that alleviating young patients from epirubicin-induced ovarian damage and sterility is possible. Furthermore, it is noteworthy that the synergistic role of melatonin has been revealed in chemotherapy-caused apoptosis in several cancer cells [24, 55, 56]. Therefore, it needs further study to explore the dual ability of melatonin.

In conclusion, this study first explored the effects of epirubicin on ovaries, identified the protective role of melatonin in ovarian damage caused by epirubicin via suppressing ERS-induced apoptosis. These data indicate that melatonin could be used to reduce the side effects of epirubicin by acting as a combination chemotherapeutic agent in young women with cancer.

Conflict of Interest: The authors declare that there are no conflicts of interest.

Acknowledgments

This study was supported by grants from the National Key R&D Program of China (2017YFC1001403), and NSFC (31871512 and 31671199) to CZ. Support was also obtained by a grant from the Major Program of the National Natural Science Foundation of China (NSFC) (81490743) to Z-J C, and by the Shanghai Commission of Science and Technology (17DZ2271100).

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