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# Polydatin Alleviates Radiation-Induced Testes Injury by Scavenging ROS and Inhibiting Apoptosis Pathways

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Statistical Analysis C  
Data Interpretation D  
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**Background:** Exposure to ionizing radiation (IR) induces severe damage in multiple human tissues. The testes are extremely sensitive to IR, and testes irradiation can result in infertility and abnormality. A novel and safe radioprotector for testes injury from IR is needed. Polydatin (PD) has been proved to have anti-oxidant and anti-inflammatory effects, indicating its potential application in radiation protection.





**Material/Methods:** Male wild-type C57BL/6 mice (8 weeks old) were exposed to ionizing radiation. At different times after irradiation, testes were isolated and subjected to hematoxylin-eosin (HE) staining and TUNEL staining, as well as related quantification. ELISA assay was used to measure the level of inflammatory cytokines, and apoptosis proteins were detected by Western blot assay. Intracellular ROS was measured by DCFH-DA flow cytometry method.

**Results:** In the present study, we demonstrated that polydatin effectively alleviated testes injury and retained sperm viability. PD pretreatment also inhibited cell apoptosis caused by irradiation. Radiation-induced decrease of FSH and testosterone was also inhibited by PD treatment. Finally, we showed that PD obviously reduced the ROS level, using DCFH-DA method. We also found that PD reduced the concentration of the oxidative products MDA and 8-OHdG. PD also inhibited apoptosis-related proteins such as Bax and caspase 3.

**Conclusions:** Our data proved that polydatin effectively alleviated testes injury after irradiation, mainly through reducing ROS and oxidative stress. Our findings suggest polydatin as a potential radioprotector for testes radiation damage.

**MeSH Keywords:** **Apoptosis • Radiation-Protective Agents • Testis**

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## Background

Exposure to ionizing radiation (IR) in a nuclear accident or disaster, dirty bomb attack, or clinical accidents can lead to acute radiation syndrome, including damage in many tissues such as bone marrow, intestines, and testes [1]. Among these, the testes are extremely sensitive to IR, and even 0.5 Gy irradiation can result in infertility and spermatogenesis dysfunction [2,3]. There is no drug available for clinical use to prevent testicular radiation damage. The only FDA approved radioprotector – Amifostine – showed severe tissue toxicity during radiotherapy [4,5]. Novel and effective radioprotectors for testes injury are urgently needed.

Polydatin is a natural extract from the root of *Polygonum cuspidatum*, and has been proved to exert anti-oxidative and anti-inflammatory effects. Recently, it was reported that polydatin alleviates radiation-induced lung injury through activation of Sirt3 [6]. As oxidative stress and inflammation are 2 main factors driving radiation damage, we are interested in whether polydatin could protect testes against radiation damage. In this study, we demonstrated that polydatin effectively attenuated radiation-induced structural and functional injury of testes, suggesting it as a potential novel radioprotector for testes.

## Material and Methods

### Mice and treatment

Male wild-type C57BL/6 mice (8 weeks old) were purchased from the laboratory of Jilin University. All mice were housed in individual cages in a temperature-controlled room in an SPF animal center. For our experiments, all mice were randomly divided into 4 groups: a normal control group, a polydatin group, an IR group, and an IR+polydatin group. Before irradiation, mice were given polydatin or saline through intraperitoneal injection at the concentration of 100 mg/Kg. All the protocols were approved by the Animal Ethics Committee of Jilin University, China, in accordance with the Guide for Care and Use of Laboratory Animals published by the US National Institute of Health (publication no. 96-01).

### Irradiation

GC-1 cells (ATCC, USA) and mice were irradiated with  $\gamma$ -rays using a  $^{60}\text{Co}$  irradiator (the Second Hospital of Jilin University, Changchun, China). After different treatments, mice were exposed to 4 Gy irradiation at the dose rate of 1 Gy/min, as previously described [7]. Mice were put in a holder designed to immobilize anesthetized mice so that the abdomen was exposed to the beam. All irradiations were performed at room temperature.

### Cells and treatments

Mice spermatogonia GC-1 spg cells (ATCC, USA) were maintained in RPMI 1640 (Hyclone, Logan, UT, USA) medium with 10% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin-glutamine (Hyclone, Logan, UT, USA) at 37°C in a 5%  $\text{CO}_2$  humidified chamber. Polydatin treatments were conducted at the concentration of 300  $\mu\text{M}$  at 0.5 h before irradiation. After that, apoptosis or ROS detection were performed.

### Tissues isolation and hematoxylin-eosin (HE) staining

At the 7<sup>th</sup> day post-irradiation, testes from both sides were isolated and fixed in 4% paraformaldehyde. Tissues sections was mounted onto the slides and an HE staining kit was used according the manufacturer's instructions (Sango Biotech, Shanghai, China).

### Testes index and sperm counts

The weight of testes and the body weight of the same mice were measured and recorded. The testes index was calculated as: TI=testes weight/body weight. For sperm counts, the right cauda epididymis of each mouse was weighed and placed in 0.5-mL PBS, and homogenized for 30 s. Then, 10- $\mu\text{L}$  aliquot of each sample was diluted and the sperm were counted with a hemocytometer [8].

### TUNEL assay

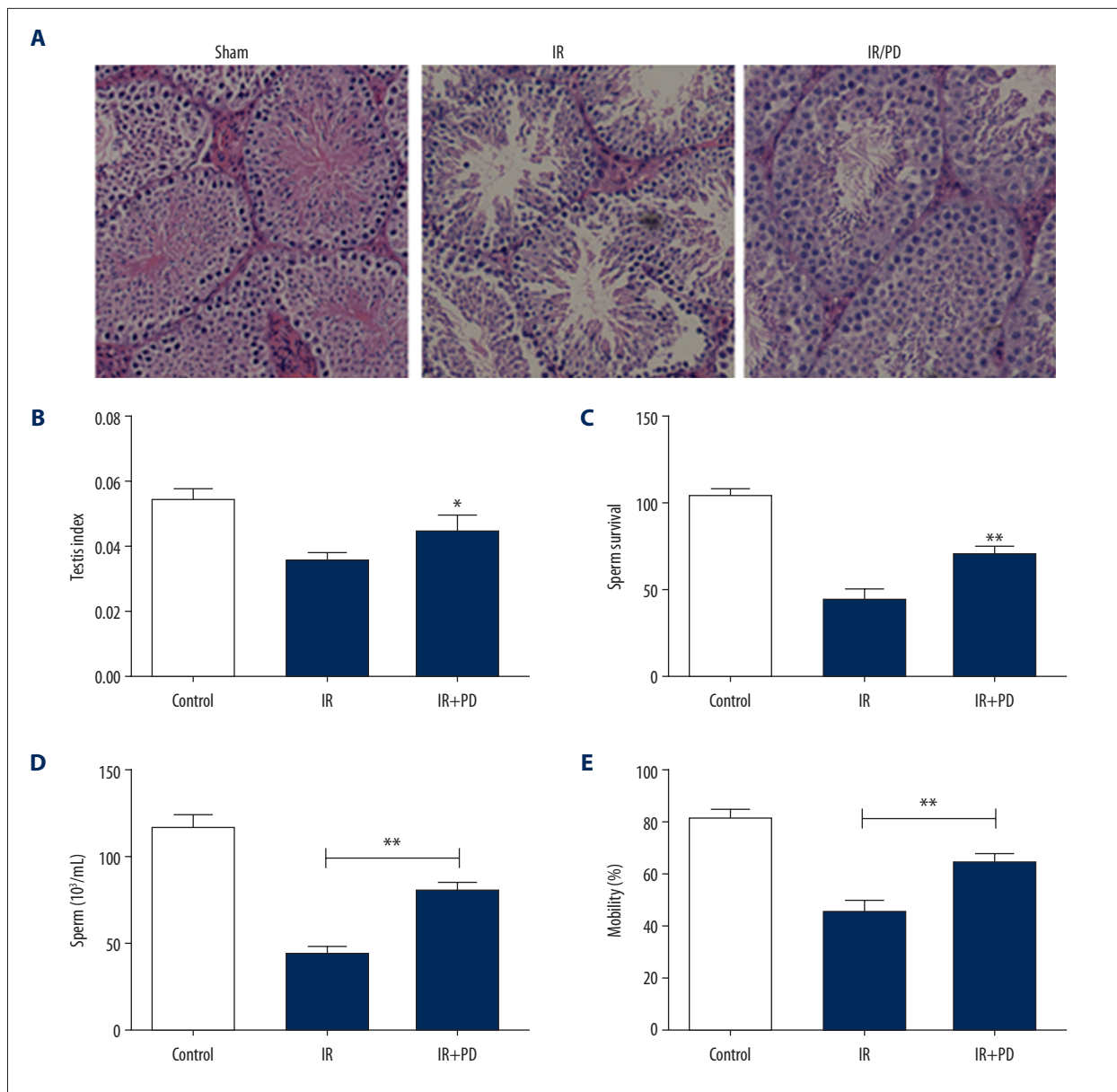
In this study, we used a TUNEL FITC Apoptosis Detection Kit (Vazyme, China) to stain the apoptotic cells in testes after irradiation, in accordance with the manufacturer's instructions.

### Flow cytometry detection of ROS

For detection of ROS, a DCFH-DA kit was purchased from Beyotime Biotech Co. Briefly, before irradiation, cells were treated with DCFH-DA dye according to the manufacturer's instructions. After irradiation, cells were analyzed with flow cytometry and the mean fluorescence was assessed.

### ELISA assay

On the 3<sup>rd</sup> day after irradiation, serum and testes were isolated and subjected to ELISA. FSH and testosterone in serum and testes were measured with a commercial ELISA kit according to the manufacturer's instructions. Inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 were also measured by ELISA, as were serum MDA and 8-OHdG.



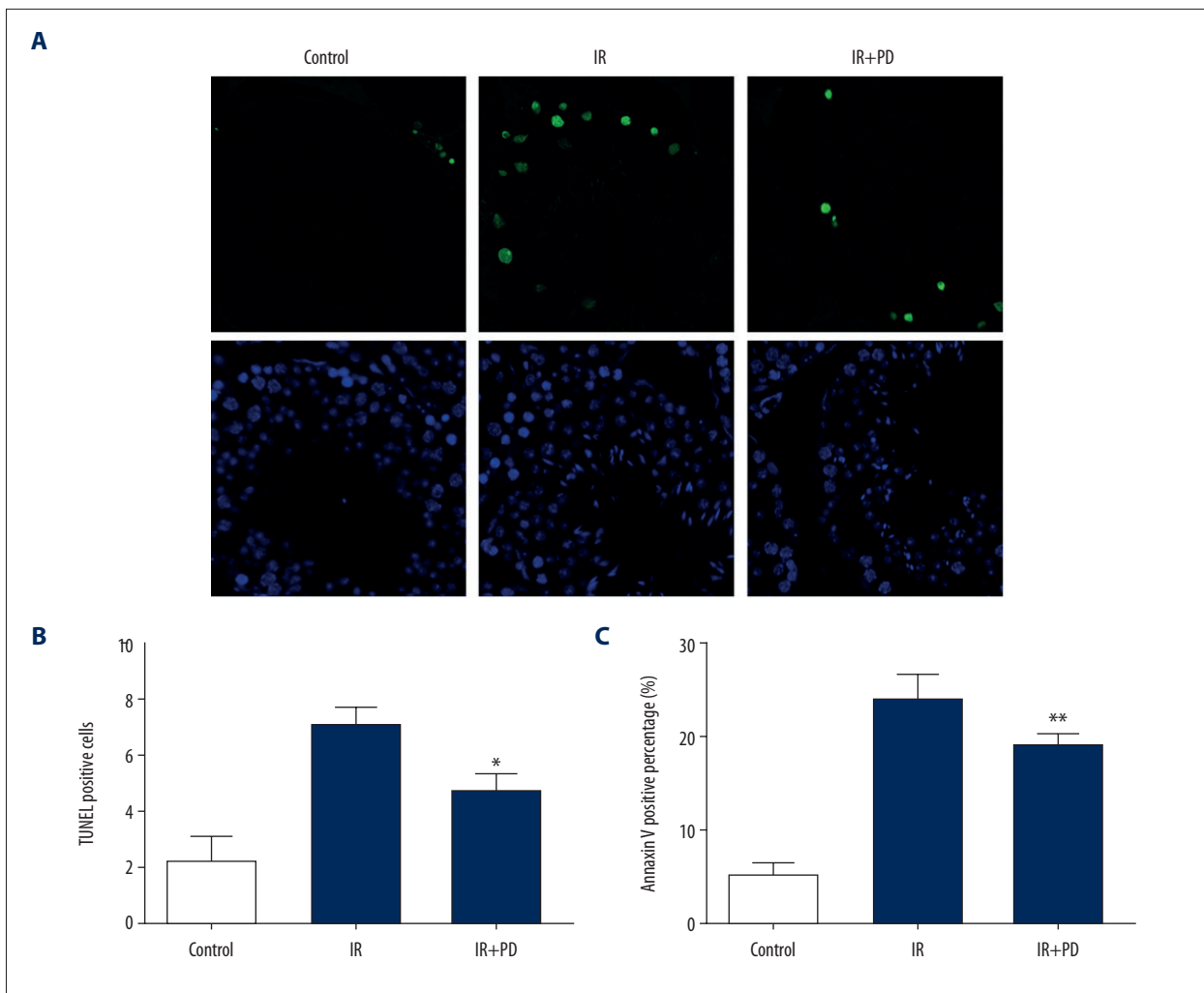
**Figure 1.** Polydatin reduced tissue injury in murine testes and increased survival sperms. **(A)** HE staining of testes in different groups isolated at 3<sup>rd</sup> day after irradiation (n=10). **(B, C)** Bar graph of testes index and sperm viability at the 3<sup>rd</sup> day post-irradiation (n=10). **(D, E)** Bar graph of sperm counts and mobility in different groups (n=10). \* P<0.05, \*\* P<0.01 compared with single-irradiation group.

### Antibodies and Western blotting

Proteins from GC-1 spg cells were extracted at 6 h after irradiation, and Western blot assay was performed, as previously described. In our study, cleaved caspase 3 (Cell Signaling Tech., 1: 1000), Bax (Cell Signaling Tech., 1: 1000), Bcl-2 (Cell Signaling Tech., 1: 1000), p53 (Cell Signaling Tech., 1: 1000), and GAPDH (Cell Signaling Tech., 1: 1000) were detected according to the protocol provided by CST. Secondary antibody was also purchased from CST.

### Statistical analysis

Data are shown as means  $\pm$  standard error of mean (SEM) for each experiment. The statistical analysis was performed using SPSS 17.0 software. Comparison between different groups were performed by using ANOVA and within groups using the *t* test. Values of P<0.05 were considered significant. The number of samples is indicated in the description of each experiment. All the experiments were repeated at least 3 independent times.



**Figure 2.** Polydatin inhibited testicular cell apoptosis after irradiation. (A) representative images of TUNEL staining of testes at 24 h after irradiation (n=10). (B) TUNEL analysis in different groups (n=10). (C) Annexin V apoptosis assay in GC-1 spg cells with different treatments (n=8). \* P<0.05, \*\* P<0.01 compared with single-irradiation group.

## Results

### Polydatin reduced tissue injury in murine testes and increased sperm quality

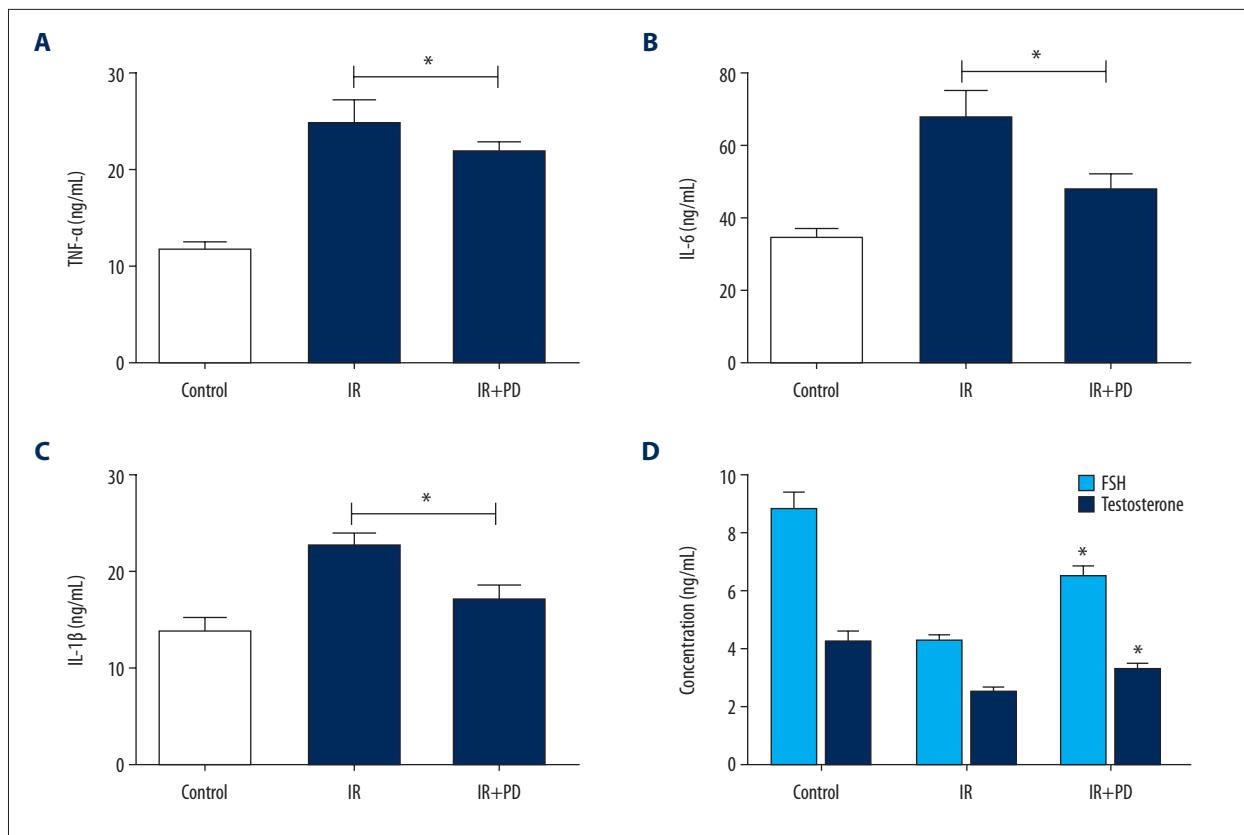
We found that polydatin treatment effectively protected testes structures against radiation damage and reduced the loss of spermatophores (Figure 1A). Polydatin also significantly inhibited testes index reduction caused by irradiation (Figure 1B, P<0.05). When checking the survival of sperm in different groups, we found that the survival of sperm decreased to  $40\pm 4.9\%$  in the single-radiation group, while in the polydatin group, the survival increased to  $70\pm 2.3\%$  (Figure 1C, P<0.01). The total number and mobility of sperms was also significantly improved (Figure 1D, P<0.01; Figure 1E, P<0.01). These data suggest that polydatin effectively alleviates radiation-induced testes injury.

### Polydatin inhibited testicular cell apoptosis after irradiation

We used TUNEL assay to determine the *in-situ* apoptosis in spermatophores in testes, showing that polydatin significantly inhibited cell apoptosis compared with the single-radiation group (Figure 2A, 2B, P<0.05). To validate this effect *in vitro*, we checked the apoptosis rate in GC-1 spg cells, and found that polydatin treatment significantly reduced the percentage of Annexin V-positive cells compared with the radiation group (Figure 2C, P<0.01).

### Polydatin inhibited inflammatory cytokines and reversed dysregulation of follicle-stimulating hormone (FSH) and testosterone against IR

Radiation induced the upregulation of inflammatory cytokines, including TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, and polydatin treatment



**Figure 3.** Polydatin inhibited inflammatory cytokines and increased the concentration of follicle-stimulating hormone (FSH) and testosterone after IR. The concentrations of TNF- $\alpha$  (A), IL-6 (B), and IL-1 $\beta$  (C) were measured by ELISA. FSH and testosterone was also measured (D). \*  $P < 0.05$ , compared with single-irradiation group ( $n = 10$ ).

significantly reduced the level of these cytokines, indicating an anti-inflammatory role (Figure 3A,  $P < 0.05$ ; 3B,  $P < 0.05$ ; 3C,  $P < 0.05$ ). In the single-radiation group, serum FSH and testosterone level decreased dramatically, but in polydatin-treated groups, the levels of FSH and testosterone were significantly elevated (Figure 3D,  $P < 0.05$ ), and the reduction of FSH and testosterone in testes tissues was also prevented. These data indicate that polydatin protects testicular functions.

#### Polydatin reduced ROS and oxidative damage in testes and cells

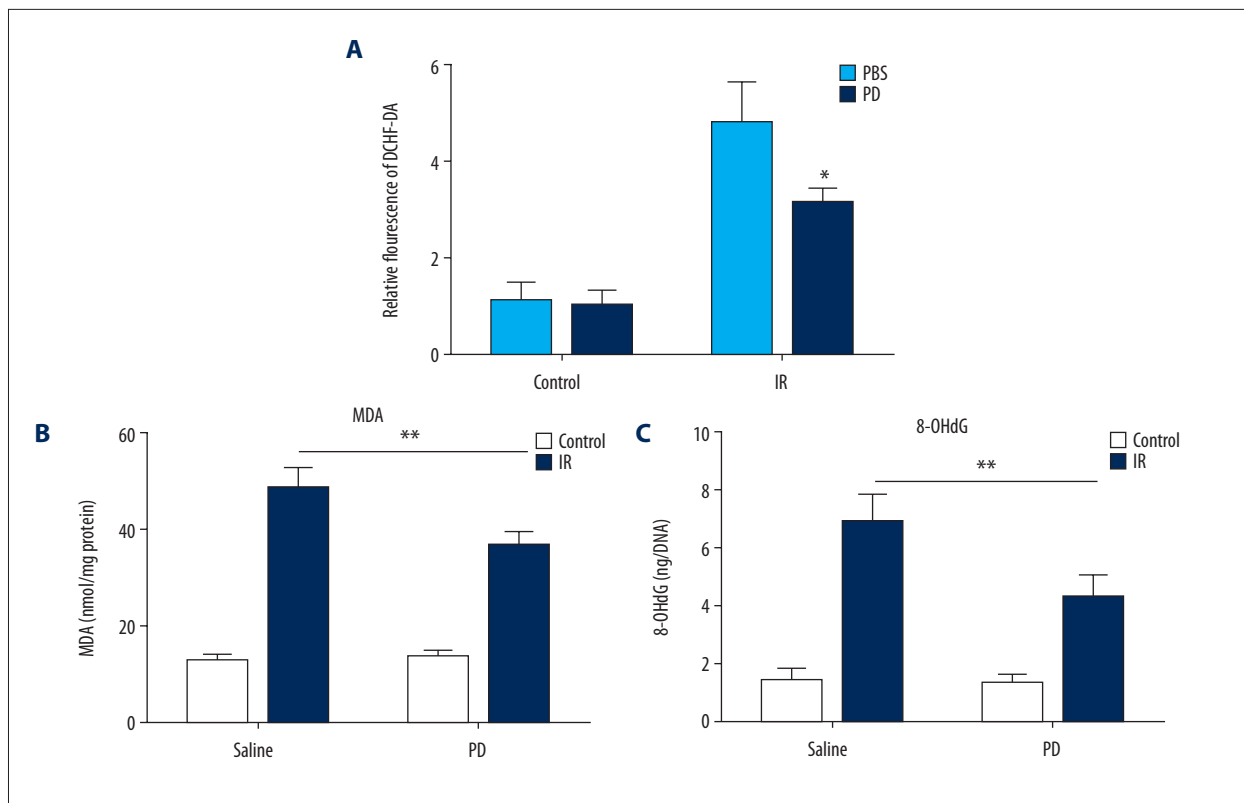
As polydatin was proved to exhibit anti-oxidant functions, we then used a DCFH-DA kit to detect the ROS level. We found that polydatin significantly reduced the ROS level in GC-1 spg cells generated by IR (Figure 4A,  $P < 0.05$ ), and the level of MDA and 8-OHdG in mouse serum were also reduced by polydatin treatment (Figure 4B,  $P < 0.01$ ; 4C,  $P < 0.01$ ).

#### Polydatin inhibited radiation activation of pro-apoptosis signaling pathway

Radiation-induced activation of cell apoptosis accounts for a major part of cellular toxicity. In our study, we found that polydatin inhibited cell apoptosis caused by irradiation (Figure 2), and we also found that polydatin treatment significantly reduced the level of pro-apoptotic protein, Bax ( $P < 0.01$ ) and cleaved caspase 3 ( $P < 0.05$ ) (Figure 5A, 5B). Moreover, the anti-apoptotic molecule Bcl-2 was significantly elevated ( $P < 0.05$ ). However, no significant difference was found in p53 expression ( $P > 0.05$ ) (Figure 5A, 5B).

#### Discussion

In this study, we demonstrated that polydatin (PD) effectively alleviated testes injury in response to ionizing radiation, preserving sperm viability and function. PD pretreatment also inhibited radiation-induced cell apoptosis *in vitro* and *in vivo*, which might account for the radioprotective effect on sperm genesis function. Radiation-induced decrease of FSH and testosterone was also inhibited by PD treatment. Finally, using



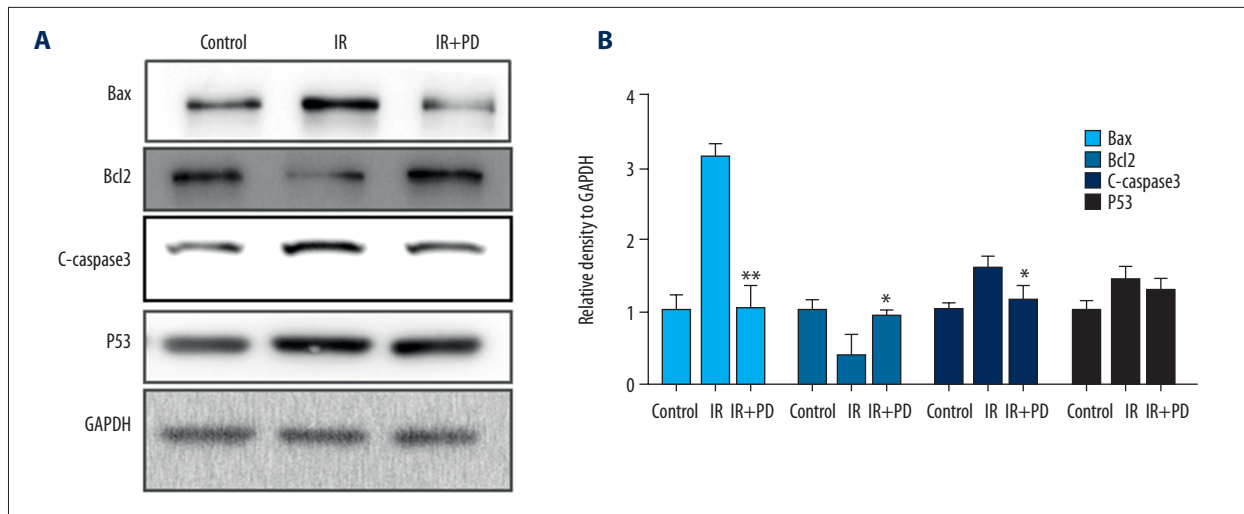
**Figure 4.** Polydatin reduced ROS and oxidative damage in testes and cells. (A) Flow cytometry analysis of ROS by using a DCFH-DA kit. (B, C) Concentration of MDA and 8-OHdG in serum after irradiation with/without PD treatment. \*  $P < 0.05$ , \*\*  $P < 0.01$  compared with single-irradiation group ( $n=10$ ).

DCFH-DA method, we showed that PD obviously reduced the ROS level. To determine the oxidative damage in testes, we checked the level of oxidative products MDA and 8-OHdG and found that PD reduced the concentration of these 2 products, confirming the anti-oxidant role of PD.

Pathological section is a direct sign of tissue damage, which reflects the cell loss, structural damage, and cellular changes [9,10]. In our study, we observed the tissue protection through HE staining, showing that radiation-induced sperm cell loss was obviously inhibited by PD treatment. An organ index has been used in evaluating tissue damage in many studies [11]. Our data showed that PD greatly increased testes index after IR, which proved the radioprotective effects of PD on testes. For functional studies, we counted live sperm and found PD increased the percentage of live sperm, which indicated that the possibility of fertility was retained. Infertility is an important outcome in people exposed to IR in nuclear accidents or medical applications [12,13]. PD also inhibited radiation-induced cell apoptosis and inhibited the apoptosis pathway, including Bax and cleaved caspase 3. A previous study also proved that polydatin inhibited radiation-induced apoptosis in lung epithelial cells [14], and another study found that PD also inhibited methylglyoxal-induced apoptosis through reducing ROS

and improving mitochondrial function in HUVEC cells [15]. However, in many cancer cells, polydatin exhibits apoptosis-promoting effects, including lung cancer cells, cervical cancer cells, and leukemia cells [16–19]. The diverse role of polydatin in cancer cells and normal cells provide a novel possibility in application in cancer radiotherapy. Mechanically, our data showed that PD inhibited radiation-induced upregulation of Bax and caspase3. However, in an osteosarcoma cells model, PD increased expression of Bax, attenuated expression of Bcl-2, and augmented caspase-3 activity [20]. Thus, the effects of PD on apoptosis pathway in different cell model is different, indicating an indirect role. The exact mechanism by which PD protects against radiation damage remains to be determined.

Ionizing radiation induces tissue damage through direct effects interacting with biological molecules, or by indirect effects such as by free radicals from radiolysis of water [21,22]. PD was reported to scavenge free radicals and to play an antioxidant role in many experimental models [23,24]. We hypothesized that the free radicals-scavenging effects of PD might account for the radioprotective effects. Through a DCFH-DA method, we proved that PD significantly inhibited ROS level, which was greatly elevated by IR. This is also an important reason why PD works effectively when given before irradiation.



**Figure 5.** Polydatin inhibited apoptotic signaling pathway. (A) Representative images of apoptotic related proteins detected by Western blotting assay. (B) The relative raw density of apoptosis proteins compared to internal control. \*  $P < 0.05$ , \*\*  $P < 0.01$  compared with single-irradiation group (n=6).

To investigate the effects of PD on oxidative injury *in vivo*, we measured the level of MDA and 8-OHdG with ELISA, and our data showed that PD reduced the concentration of MDA and 8-OHdG, which proves that PD plays an anti-oxidative role in the testes. Other groups also demonstrated that PD regulates oxidative stress pathways in other models. For example, Qiao et al. reported that polydatin alleviates  $H_2O_2$ -induced oxidative stress through the PKC pathway [25]. Polydatin was also shown to protect against ethanol-induced liver injury through regulating Nrf-2 and TLR4-NF- $\kappa$ B pathway [26]. In a rat model of osteoarthritis, Yang et al. proved that polydatin significantly reduced inflammatory cytokine levels, indicating an effective anti-inflammatory function [27].

Polydatin is extracted from the root of *Rhizoma polygoni cuspidati*, and it is a natural precursor of resveratrol [28]. It has been reported that resveratrol protects normal tissues against ionizing radiation, including lung tissues, the intestines, and lymphocytes [29,30]. Resveratrol also protects against testes injury caused by azoxymethane or high-fructose exposure [30,31]. However, there is no report on the radioprotective effects on the testes in either polydatin or resveratrol. Polydatin is a

glycoside of resveratrol, and the conformational changes result in advantageous biological properties. Polydatin is more efficiently absorbed and more resistant to enzymatic oxidation than is resveratrol [33]. Compared with resveratrol, which penetrates cells passively, polydatin enter cells via an active mechanism using glucose carriers [33]. These properties give polydatin better bioavailability than resveratrol. Our study proves that polydatin effectively protects the testes against ionizing radiation, suggesting it as a novel testicular radioprotector.

## Conclusions

Our results demonstrated that PD effectively protected against testes damage from ionizing radiation. PD significantly inhibited cell apoptosis and reduced ROS level. The oxidative products in testes was also reduced. These data suggest that PD could be a potential radioprotector for protecting the testes against IR.

## Conflicts of interest

None.

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