Original Article

Low-dose ionizing irradiation triggers apoptosis of undifferentiated spermatogonia *in vivo* and *in vitro*

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Background: The present study aimed to investigate the mechanism of low-dose ionizing radiation (IR) induced apoptosis of undifferentiated spermatogonia *in vivo* and *in vitro*.

Methods: Following 50 mGy IR, testicular tissues were collected from the adult DBA/2 mice at 1, 2 and 24 h; mice in the control group received pseudo-irradiation. Immunofluorescence (IF) staining and TUNEL were performed to assess DNA damage and apoptosis, respectively, in the irradiated testicular tissues. Furthermore, the spermatogonia were also irradiated *in vitro*, and the expression of apoptosis-related proteins was detected by Western blotting. TUNEL and flow cytometry were applied to assess cell apoptosis.

Results: γH2AX (a marker of DNA damage) was up-regulated in the seminiferous tubules at 1 and 2 h after IR, but it was reduced following the DNA repair. This was consistent with the finding that apoptosis of germline cells was present in the seminiferous tubules after IR, especially at 1 h (IF and TUNEL). Apoptosis was also present in the PLZF(+) spermatogonia, particularly at 1 h after IR. Apoptotic cells decreased with the increase in DNA repair time after IR. Moreover, the caspase-3 protein was expressed in the undifferentiated spermatogonia following IR. The expression of caspase-3, P53, Ku70 and DNA-PKcs in the cultured spermatogonia was also up-regulated following IR *in vitro*, but their expression decreased gradually over time after IR, which was supported by the findings from flow cytometry, and the apoptosis of spermatogonia peaked at 24 h post IR.

Conclusions: IR may induce the apoptosis of spermatogonia at early stage *in vivo*, but the apoptosis of spermatogonia secondary to IR occurs at a relatively later time point (24 h) *in vitro* mainly. The apoptosis of spermatogonia is improved over time after IR.

Keywords: Undifferentiated spermatogonia; apoptosis; irradiation

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Introduction

DNA damage is widely observed in the eukaryotic cells. They are two types of DNA damage: single strand break (SSB) and double strands break (DSB). Many factors contribute to DNA DSB, including reactive oxygen species (ROS), formation of replication forks during cell replication and ionizing irradiation (IR). IR causes cell genomic DNA DSBs, which directly affects cell genomic stability. DSBs have been reported to trigger the most detrimental effects on the genome stability and identified as the main contributor to IR-induced cell-killing effect through the formation of chromosomal aberrations. Studies

have shown that IR may adversely affect male germ cells, and further influences male spermatogenesis (1,2). The male testicular germ cells are more sensitive to DNA DSB than other tissues (3). Radiotherapy, chemotherapy and other treatments for tumors may also cause DNA damage of germ cells, resulting in infertility (4,5). Undifferentiated spermatogonia (As, Apr and Align) is a unique spermatogonia in adult male testes and plays an important role in maintaining fertility and spermatogenesis. Although it has been reported that low-dose IR may cause DNA DSB in the spermatogonia, the relationship between low-dose IR and apoptosis of undifferentiated spermatogonia/progenitor cells is still poorly understood. Therefore, the present study aimed to investigate the low-dose IR induced DNA DSB and explore the relationship between the DNA DSB and the apoptosis in undifferentiated spermatogonia *in vivo* and *in vitro*.

Methods

Establishment of germline cell DNA damage mouse model

This study was approved by the Ethics Committee of Tongji Hospital. Twelve one-month-old DBA/2 male mice were divided into four groups, according to DNA damage-repair time (1, 2, 24 h and control). The total dose of irradiation (Mark I-68A, JL Shepherd and Associates, San Fernando, CA, USA; irradiation condition: 0.1 Gy/min for 30 s) was applied in the radiation groups, and in the control group, mice were received pseudo-irradiation. The testes were collected at different time points of DNA repair (1, 2, 24 h) after IR. The testicular tissues were fixed in 4% paraformaldehyde and then sectioned (10 μm in thickness).

Immunofluorescence staining of testicular sections

The testicular tissue sections were blocked in a solution containing 2% BSA, 0.05% Tween 20 and 0.1% Triton X-100 at 4 ℃ overnight, and then incubated with mouse anti-γH2AX (Millipore, 05-636, Burlington, USA) at 1:200, rabbit anti-promyelocytic leukemia zinc finger (PLZF; Santa Cruz, sc-22839, Dallas, USA) at 1:200, anti-caspase (Abcam, ab13847, Cambridge, MA, USA) at 4 ℃ overnight. Subsequently, the sections were incubated with secondary antibodies at 1:200 overnight at 4 ℃. The secondary antibodies were rabbit anti-mouse IgG (H+L) superclonal antibody, Alexa Fluor 555 (Invitrogen, A27028, Waltham, USA), goat anti-rabbit IgG (H+L) highly cross-absorbed antibody, Alexa Fluor 488 (Invitrogen, A-11034, Waltham, USA). Cell nuclei were counter-stained with 0.3% DAPI (Beyotime Biotechnology, Shanghai, China). Sections were rinsed with PBS containing 0.05% Tween 20 and then mounted with anti-fade mounting medium.

TUNEL staining of germ cells in testicular tissue sections

Proteinase K working solution was diluted with PBS at a final concentration of 20 μg/mL. Each section was treated with 100 μL of proteinase K working solution at room temperature (RT) for 10 min. After rinsing with PBS for 2–3 times, each section was incubated with 100 μL of 1× equilibration buffer for 10–30 min at RT, followed by addition of a solution containing 50 μL of TdT enzyme. Then, the sections were incubated at 37 ℃ for 60 min, followed by rinsing with PBS containing 0.1% Triton X-100 and 0.05% Tween20. Subsequently, anti-PLZF (Santa Cruz, sc-22839, Dallas, USA) antibody at 1:200 was added to each sample, followed by incubation at 4 ℃ overnight. On the next day, the sections were rinsed with PBS containing 0.1% Triton X-100 and 0.05% Tween20, and then incubated with secondary antibody at RT for 2 h. The nuclei were counter-stained with 0.3% DAPI solution.

Culture and proliferation of mouse spermatogonia

The primary germline stem cells were collected and cultured with the procedures reported in our previous study (6). In brief, the testes were collected from 4-week-old male DBA/2 mice and then treated with Collagenase type IV at 1 mg/ml (Sigma, C5138, St. Louis, USA) and 0.25% Trypsin (Gibco, 25200072, Waltham, USA) to release the testicular cells. THY-1 (CD90) positive germ cells were sorted with the magnetic bead sorting system (MACS). The sorted cells were transferred to CF1 MEF (mouse embryonic fibroblast) feeder cells for further culture. The undifferentiated spermatogonia cells were cultured in the medium containing StemPro-34 (Life Technologies, Carlsbad, CA, USA, 10639-011), StemPro-34 supplement at 20 μL/mL (Life Technologies, 10639-011), insulin at 25 μg/mL (Sigma, 13536), transferrin at 100 μg/mL (Sigma, T1147), pyruvic acid at 100 μg/mL (Sigma, P5280), putrescine at 60 μM (Sigma, P5780), sodium selenite at 30 nM (Sigma, S5261), D-(+)-glucose at 6 mg/mL (Sigma, 49139), lactic acid at 1 μL/mL (Sigma, L4263), L-glutamine at 2 mM (Life Technologies, 25030-081), 2-mercaptoethanol 5×10^{-5} (Merck Millipore, Darmstadt,

Germany, ES-007-E), minimal essential medium (MEM) vitamin solution 19 (Life technologies, 11120-052), MEM non-essential amino acid solution 19 (Life Technologies, 11120-052), 10-4 M ascorbic acid (Sigma, A4403), d-Biotin at 10 μg/mL (Sigma, 14400), b-estradiol at 30 ng/mL (Sigma, E2758), progesterone at 60 ng/mL (Sigma, P5096), human basic fibroblast growth factor at 10 ng/mL (Life Technologies, 13256-029), murine leukemia inhibitory factor at 103 U/mL (Merck Millipore, Temecula, CA, USA, ESG1107), recombinant human glial cell line-derived neurotrophic factor (GDNF) at 10 ng/mL (Life Technologies, PHC7041), and 1% fetal calf serum (FCS; Life Technologies, 10099- 141).

Western blotting for detecting apoptosis-related proteins in undifferentiated spermatogonia

Spermatogonia cells were collected at different time points after IR. The cells were lysed with RIPA lysis buffer containing 1 mM proteinase inhibitor PMSF. BCA protein assay kit (Beyotime Biotechnology, Shanghai, China) was used to determine the protein concentration. Protein samples were separated by 10–12% SDS-PAGE and then transferred onto 0.45-μm PVDF membrane (Millipore, Burlington, USA), which was then incubated with specific antibodies: P53 (Abcam, PAb 240), p-P53 (Ab1431), Ku70 (Santa Cruz, sc-365766), DNA-PKcs (Ab32566), caspase-3 and GAPDH (SC-32233).

Detection of apoptosis in undifferentiated spermatogonia by flow cytometry

The cultured germline cells were irradiated at 50 mGy, and then collected at pre-designed time points. To remove MEF cells, the cell suspension was digested with 0.05% trypsin solution and then transferred to 0.2% gelatin coated dish, followed by incubation for 2 h. The floating cells were collected and rinsed with PBS. Annexin V (BD) and propidium iodide (PI) were added for staining. After 15-min incubation, cells were subjected to flow cytometry.

Results

Low-dose IR induced DNA DSB in germ cells of testicular seminiferous tubules

IF staining indicated the expression of γH2AX (a marker of DNA DSB) in the germ cells of mouse testicular seminiferous tubules after IR (*Figure 1*). γH2AX was highly expressed in the seminiferous tubules, especially at 1 and 2 h after IR, and its expression decreased over time after IR, suggesting that the DNA damage is repaired by germline cells (*Figure 2*).

Low-dose IR triggered germline cells apoptosis in seminiferous tubules of mouse testes

TUNEL showed TUNEL positive germline cells in the testicular seminiferous tubules after IR (*Figure 3*). Apoptotic germline cells increased significantly after IR as compared to the control group ($P<0.05$). The apoptosis of germ cells peaked at 24 h after IR (*Figure 4*).

Low-dose IR induced DNA damage triggered apoptosis of undifferentiated spermatogonia in testicular seminiferous tubules

PLZF double staining TUNEL was employed to investigate the apoptosis of PLZF(+) undifferentiated spermatogonia in which the progenitor spermatogonia was positive to PLZF. Results showed the apoptotic undifferentiated spermatogonia were also found in the testicular seminiferous tubules after IR. Moreover, the apoptosis of PLZF(+) spermatogonia peaked at 1 h after IR (*Figures 3*,*5*). Similarly, the caspase-3 expression was also observed in the PLZF(+) cells after IR, even at 24 h after IR (*Figure 6*).

Apoptosis-related proteins expression increased in undifferentiated spermatogonia in vitro post IR

The expression of Ku70 (a marker of DNA damage) and DNA-PKcs was up-regulated in the undifferentiated spermatogonia after IR, suggested that low-dose IR also induces DNA damage in the progenitor spermatogonia *in vitro*. The expression of apoptosis-related proteins P53 and p-P53 was also up-regulated in the undifferentiated spermatogonia. Caspase-3 expression increased consistently after IR, and the cleaved caspase-3 expression also increased significantly as compared to the control group after IR ($P<0.05$) (*Figure 7*).

Apoptotic undifferentiated spermatogonia increased in vitro after IR

Flow cytometry was done to determine the apoptotic undifferentiated spermatogonia after IR. Results showed

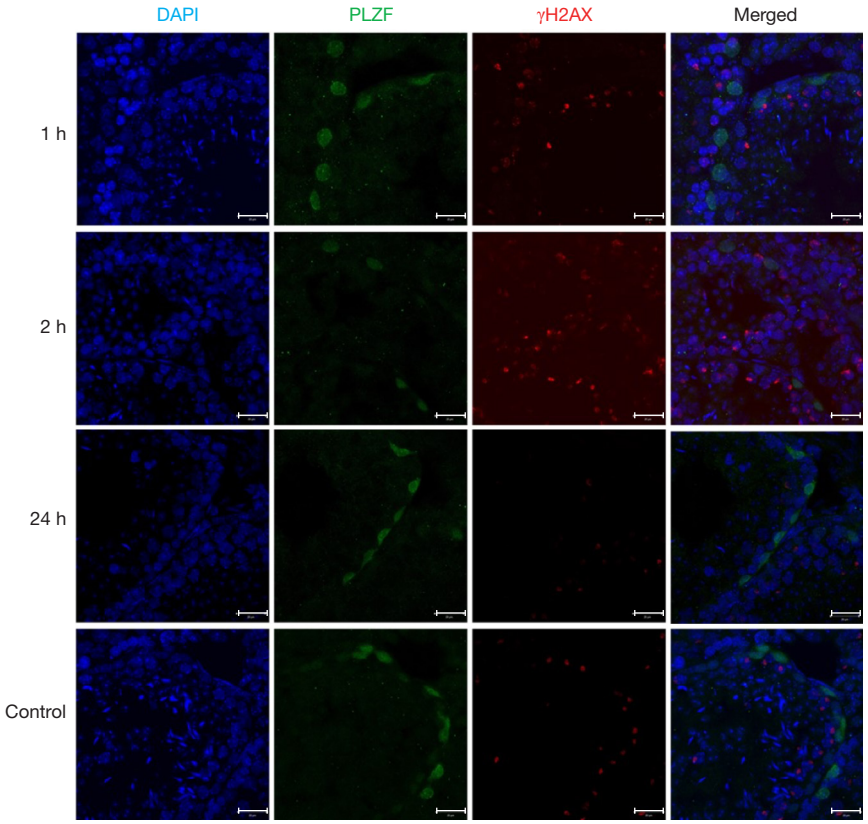

Figure 1 γH2AX expression in the seminiferous tubules after low-dose IR. Testicular tissues were collected at different time points (1, 2 and 24 h). γH2AX expression was observed in the tissues treated with IR; the high intensity γH2AX signals represented the germline cells at meiosis stage. γH2AX signals were up-regulated at 1 and 2 h after IR. PLZF positive spermatogonias were mainly found in the basal region of the seminiferous tubules. Scale bar, 25 μm.

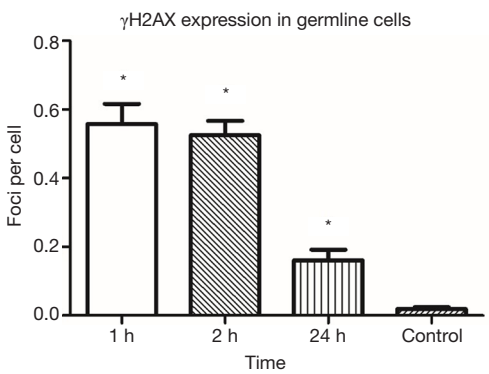

Figure 2 γH2AX expression in the germline cells after IR *in vivo*. The γH2AX expression was statistically analyzed. γH2AX expression was significantly unregulated as compared to the control group. γH2AX expression decreased over time after IR, suggesting that the DNA DSBs were repaired in the germline cells. More than 10 sections were examined, and more than 200 cells were counted (*, P<0.05 *vs.* control group). IR, ionizing irradiation; DSB, double strands break.

that apoptosis peaked at a relatively later time point (24 h). Apoptotic cells dramatically increased after IR as compared to the control group, including the early stage (annexin V positive, PI negative) and late stage (annexin V positive, PI positive) apoptotic undifferentiated spermatogonia (P<0.05) (*Figures 8,9*).

Discussion

DNA damage is common in the eukaryotic cells. The most serious effect of IR on the eukaryotic cells may be DNA DSB and genomic instability (7,8). Many cancer patients usually receive radiotherapy, and the dose of radiation is often higher than that used in animal studies (9). Currently, radiological examination and treatments are widely used in clinical diagnosis and treatment. The precise mechanism of biological effects of irradiation on the undifferentiated

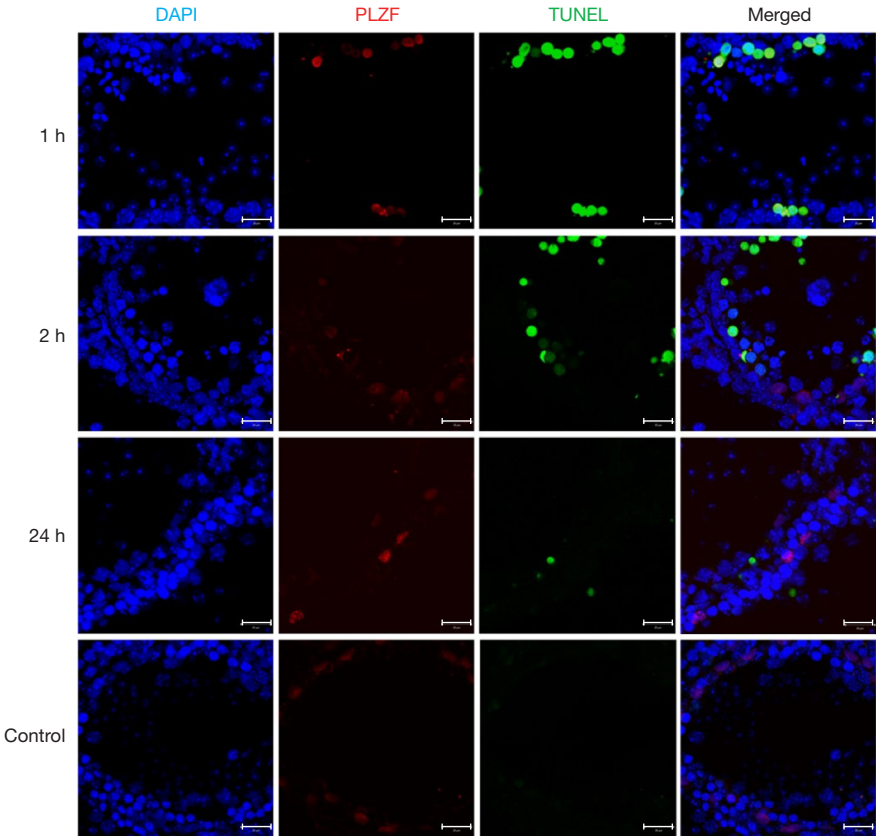

Figure 3 Apoptotic cells in the seminiferous tubules after IR. TUNEL staining was applied to detect the apoptosis of germline cells after IR. TUNEL staining showed the apoptotic germline cells increased significantly after IR and TUNEL positive signals were still observed at 24 h. Some TUNEL positive cells were also found in PLZF(+) spermatogonia, and the apoptosis of germline progenitor cells peaked at 1 and 2 h after IR. IR, ionizing irradiation. Scale bar, 25 μm. IR, ionizing irradiation.

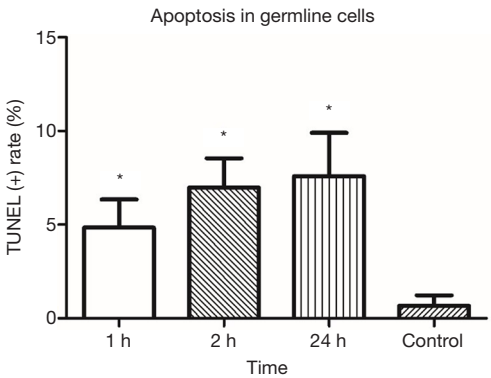

Figure 4 Apoptosis of germline cells after IR. TUNEL positive cells were analyzed in different groups. After IR, the apoptosis rates of germline cells increased as compared to the control group. More than 10 sections were examined, and more than 200 cells were counted (*, P<0.05 as control group). IR, ionizing irradiation.

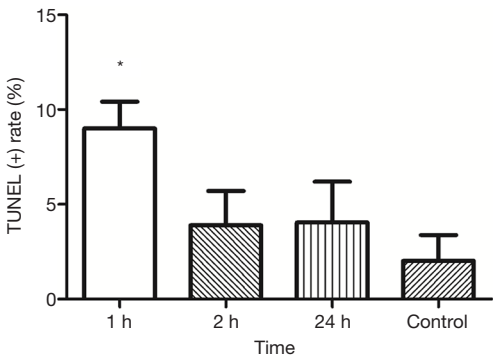

Figure 5 Apoptosis of undifferentiated spermatogonia after IR. TUNEL positive cells were counted in PLZF positive spermatogonias. The apoptosis of undifferentiated spermatogonia peaked at 1 h after IR. TUNEL positive cells were still observed at 2 and 24 h, however, the number of TUNEL positive cells was similar to that in the control group. More than 10 sections were examined, and more than 200 cells were counted (*, P<0.05 *vs.* control group). IR, ionizing irradiation.

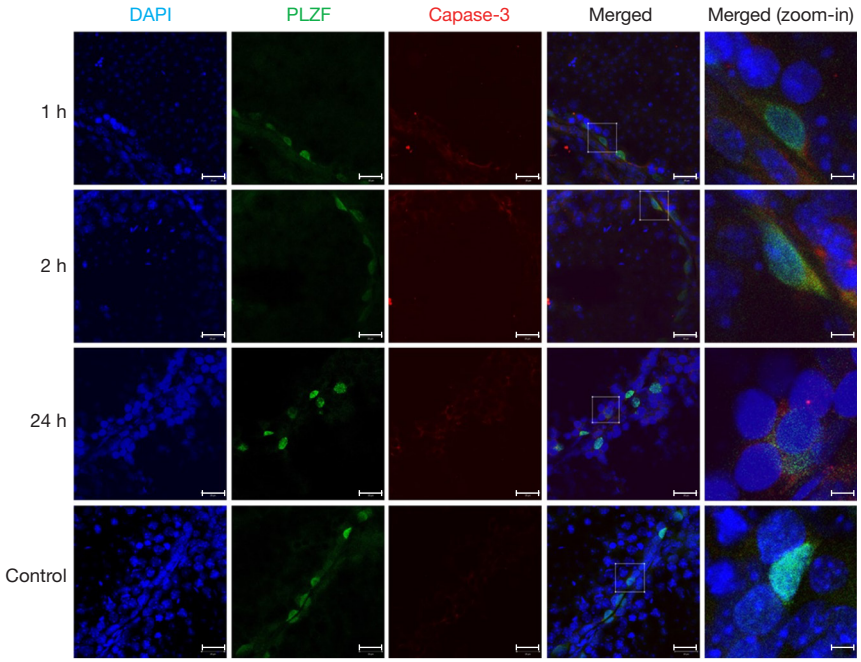

Figure 6 Caspase-3 expression in the undifferentiated spermatogonia after IR. IF staining was performed to detect the apoptosis-related protein (caspase-3) expression in PLZF(+) spermatogonias after IR. Caspase-3 expression was observed in PLZF(+) cells, mainly in the membrane of seminiferous tubules, after IR, while caspase-3 expression was not observed in the control group. Caspase-3 expression was still observed at 24 h after IR. Scale bar, 25 μm. IR, ionizing irradiation; IF, immunofluorescence.

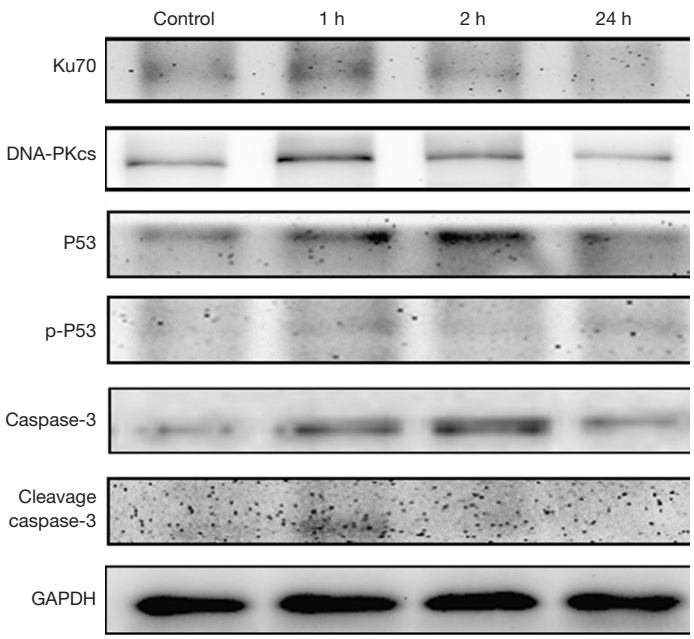

Figure 7 Apoptosis related protein expression in undifferentiated spermatogonia after IR. The expression of DNA DSBs related protein, Ku70 and DNA-PKcs, was up-regulated after IR. Similarly, the expression of apoptosis-related proteins P53, phosphorylated P53 (S15) and caspase-3 was up-regulated in the undifferentiated spermatogonias at different time points (1, 2 and 24 h) after IR. The expression of cleaved caspase-3, active form of caspase-3, was up-regulated at 1 h after IR. IR, ionizing irradiation; DSB, double strands break.

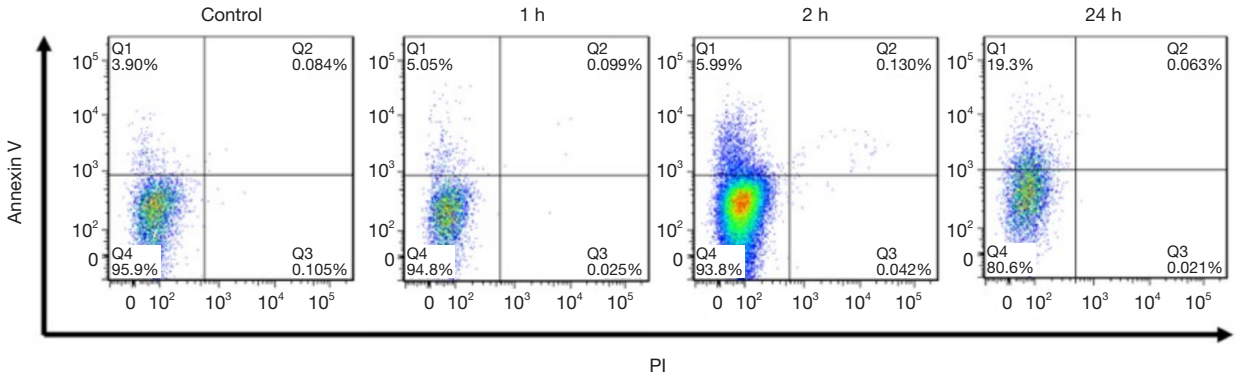

Figure 8 Flow cytometry of undifferentiated spermatogonia after low dose IR. After irradiation, cells were stained with annexin V and PI, followed by flow cytometry for the detection of apoptosis. Apoptosis of undifferentiated spermatogonias was classified as early-stage (annexin V positive, PI negative) and late-stage (annexin V positive, PI positive) one. IR, ionizing irradiation; PI, propidium iodide.

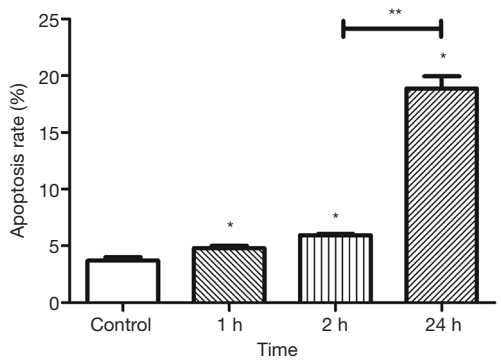

Figure 9 *In vitro* apoptosis of undifferentiated spermatogonia after IR. Apoptosis of spermatogonia was analyzed by flow cytometry. Apoptosis rate increased after IR as compare to control group. The apoptosis of spermatogonia was more obvious in 24 h group as compared to other two IR groups. (*, P<0.05 *vs.* control group; **, P<0.05 *vs.* 2 h group). IR, ionizing irradiation.

germline cells is still unclear, although studies have shown that DNA damage can affect spermatogenesis and is related to male infertility (5,10).

In the present study, THY-1 was used as a MACS marker to sort the undifferentiated spermatogonia. It has been reported that there is only a small proportion of undifferentiated germline cells in the adult mice testes (11). THY-1 is a specific surface marker specifically expressed on As, Apr and Align spermatogonia, and it has been recognized as a marker commonly used for fluorescence-activated cell sorting (FACS) or MACS sorting of undifferentiated spermatogonia (12). In some studies, THY1 is used as a cell surface marker for sorting undifferentiated spermatogonia (13,14). Although not all the undifferentiated spermatogonias are THY-1 positive, THY-1 is still an ideal indicator of progenitor spermatogonia (12,15). In the present study, undifferentiated spermatogonias were successfully isolated and purified *in vitro* by using THY-1 as a surface marker as previously reported (14,16).

PLZF is expressed in the nucleus of progenitor germline cells and has been recognized as another marker of undifferentiated spermatogonia (17). PLZF is indispensable for undifferentiated spermatogonia (especially spermatogonial stem cells) in maintaining stemness and self-renewal. PLZF deficiency may cause progressive loss of germline cells, leading to infertility in mammalians (18). Previous studies have demonstrated that PLZF expression gradually decreases during the process of Align spermatogonia, suggesting that PLZF not only plays an important role in the biological function of spermatogonial stem cells, but also is a convincing marker of undifferentiated spermatogonia. Several studies have applied PLZF as a marker to study progenitor germline cells (19,20).

γH2AX has been widely recognized as a classical marker of DNA damage (21,22). ATM promotes the phosphorylation of γH2AX at specific sites to form γH2AX after DNA DSBs (23). The γH2AX expression is helpful to evaluate the extent of DNA damage (24). IF staining of γH2AX showed that γH2AX was still expressed on the spermatogenic cells of the seminiferous tubules after low-dose IR, suggesting that the IR at a rational dose in this study still causes DSBs in the germline cells. Our previous studies revealed that the DNA repair response in the germline stem cells was a unique process independent of

γH2AX (6). In the present study, results showed PLZF(+) spermatogonias were negative to γH2AX, which is consistent with our previous results. Other studies imply that differentiated germ cells may undergo different repair mechanisms after IR (25). Studies have revealed that γH2AX can interact with P53 after IR to induce the apoptosis of differentiated spermatogonia, which is independent of DNA-PKcs (26). Moreover, Ku70, an important DNA damage repair protein, is also reported to be absent in early meiotic cells after formation of DNA breaks (27). However, our study revealed that both Ku70 and DNA-PKcs were expressed on the undifferentiated spermatogonia while H2AX expression was absent, which suggests that the undifferentiated spermatogonias experience a unique mechanism after DSB. That is, there is a cell-tissue specific mechanism for the undifferentiated spermatogonia. Previous studies have reported that the DNA repair in the germline stem cells after IR was independent of γH2AX, but it was a 53bp1 dependent process (28). Our results also confirmed that the undifferentiated spermatogonias were negative to γH2AX during the DNA damage repair, but the differentiated spermatogonias were positive to γH2AX, which was consistent with previously reported. These findings confirmed that the mechanism of DNA repair after DSB in the undifferentiated spermatogonias is different from that in other cell types, suggesting a cell-specific mechanism of DNA repair in undifferentiated spermatogonias.

In present study, TUNEL was applied to detect the apoptotic cells in the testes. It indicated that low-dose IR also induced apoptosis of spermatogenic cells in the seminiferous tubules. By double staining of PLZF and TUNEL, the TUNEL positive cells were examined in the undifferentiated spermatogonia. Our results revealed that low-dose IR contributed to the apoptosis of undifferentiated germline cells which peaked at the acute stage of DNA damage *in vivo*.

In addition, low-dose IR also induced the expression of apoptosis-related proteins in the cultured undifferentiated spermatogonia. The expression of P53 and its active form p-P53 significantly increased after IR, which remained at 24 h. Caspase-3 protein expression was also up-regulated in the spermatogonia after IR, which continued to 24 h after IR. The expression of cleaved caspase-3 was observed only in the acute phase (1 and 2 h) of DNA damage post IR.

In order to further verify the apoptosis of undifferentiated spermatogonia *in vitro*, flow cytometry was done to detect the apoptosis of IR treated cells. Results showed apoptosis occurred immediately after IR, and continued to 24 h.

It has been reported that low-dose IR may also cause DNA damage and induce DNA repair response in mammalian stem cells (29,30). In the present study, low-dose cesium (Cs) irradiation was applied to establish cell and animal DNA damage models.

Although several experiments applied X-ray to induce DNA damage both *in vivo* and *in vitro*, both gamma-ray and beta-ray can be emitted by the ^{137}Cs irradiation resource, which is a major difference in the irradiation ray between ^{137}Cs and linear accelerator. Many studies have investigated the effects of IR on the murine testes, but the doses of X-ray used in available studies still remain high as compared to that in our study (25,31).

As compared to the other studies about the effects of irradiation on the germline cells *in vivo* (32), low-dose IR was employed in our study, and results showed it could trigger the apoptosis of undifferentiated spermatogonia both *in vivo* and *in vitro*, which suggests that undifferentiated spermatogonias are more vulnerable and sensitive to DNA damage.

The *in vivo* and *in vitro* findings indicated that low-dose IR could induce the apoptosis of undifferentiated germline cells *in vivo*, which occurred mainly in the acute stage after IR, but the spermatogonia apoptosis mainly occurred at 24 h after IR *in vitro*. Moreover, caspase-3 was involved in the process of apoptosis in the progenitor germline cells both *in vivo* and *in vitro*. The cleaved caspase-3 was mainly expressed in the acute phase after IR, while flow cytometry indicated a large number of apoptotic cells at 24 h. Our findings suggested the germline cells apoptosis at different time points after IR, but more studies are needed to investigate the exact mechanism.

In summary, our results indicate that low-dose IR causes DNA damage to undifferentiated spermatogonia *in vivo*, and induce apoptosis of undifferentiated germline cells in a caspase-3 dependent manner. Decreasing progenitor/germline stem cells are an important factor of male infertility. Thus, the apoptosis of undifferentiated germline cells is also crucial for the compromised development and quantity of sperms. Our results provide direct evidence on the low-dose IR induced apoptosis of mouse undifferentiated spermatogonia, which further demonstrate that the undifferentiated spermatogonias are sensitive to DNA damage. Our findings further suggest that the apoptosis of progenitor germline cells after IR changes over time. The apoptosis of undifferentiated spermatogonia occurs mostly at a longer time after IR

in vitro as compared to that *in vivo*. The precise mechanism underlying the apoptosis of undifferentiated spermatogonia should be further elucidated. To date, few studies have been conducted to investigate the effects of low-dose IR on the germline stem cells. Our results provide evidence that IR causes apoptosis of undifferentiated spermatogonia cells and present a theoretical basis for the prevention and preservation of male infertility in the clinical radiation related treatments.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The present study was approved by the Ethics Committee of Tongji Hospital (No. KYSB-2017-102).

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