Repetitive exposure to low-dose X-irradiation attenuates testicular apoptosis in type 2 diabetic rats, likely via Akt-mediated Nrf2 activation

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Abstract

To determine whether repetitive exposure to low-dose radiation (LDR) attenuates type 2 diabetes (T2DM)-induced testicular apoptotic cell death in a T2DM rat model, we examined the effects of LDR exposure on diabetic and age-matched control rats. We found that testicular apoptosis and oxidative stress levels were significantly higher in T2DM rats than in control rats. In addition, glucose metabolism-related Akt and GSK-3 β function was downregulated and Akt negative regulators PTP1B and TRB3 were upregulated in the T2DM group. Superoxide dismutase (SOD) activity and catalase content were also found to be decreased in T2DM rats. These effects were partially prevented or reversed by repetitive LDR exposure. Nrf2 and its downstream genes NQO1, SOD, and catalase were significantly upregulated by repetitive exposure to LDR, suggesting that the reduction of T2DM-induced testicular apoptosis due to repetitive LDR exposure likely involves enhancement of testicular Akt-mediated glucose metabolism and anti-oxidative defense mechanisms.

Key words: Type 2 diabetes; testis; apoptosis; oxidative stress; nuclear factor erythroid 2-related factor 2;

Non-standard abbreviations and acronyms

LDR, low-dose radiation; STZ, streptozotocin; ROS, reactive oxygen; RNS, nitrogen species; SOD, superoxide dismutase; Nrf2, nuclear factor erythroid 2-related factor 2; NQO-1, NAD(P)H:quinone oxidoreductase; CAT, catalase; T2DM, type 2 diabetes; HFD, high-fat diet; MDA, malondialdehyde; 4-HNE, 4-hydroxynonenal; AIF, apoptosis inducing factor; 3-NT, 3-nitrotyrosine; PTP-1B, protein tyrosine phosphatase-1B; TRB3, Tribbles Homologue3; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; TdT, terminal deoxynucleotidyl transferase

1. Introduction

The global increase in the prevalence of diabetes presents significant clinical challenges due to the high rates of diabetic complications and mortality associated with the disease. For instance, according to the National Diabetes Statistics, 29.1 million American individuals, representing 9.3% of the American population, had diabetes in 2014. Furthermore, it is estimated that about 208,000 Americans under the age of 20, representing approximately 0.25% of the American population, have been diagnosed with diabetes.

(<http://www.diabetes.org/diabetes-basics/statistics/#sthash.XrouwO0y.dpuf>). It is known that diabetes is associated with pathological and functional damage to various organs, resulting in a variety of complications. Therefore, the development of efficient approaches to prevent or postpone the development of these complications is critical. Diabetes is significantly associated with infertility in males (Dinulovic and Radonjic, 1990). Although several mechanisms are considered to play a role in the development of infertility in diabetic men (Agbaje et al., 2007; Amaral et al., 2008; Dinulovic and Radonjic, 1990), germ cell loss may represent the direct and most important contributor to the loss of fertility in diabetic males (Cai et al., 2000; Koh, 2007; Sainio-Pollanen et al., 1997).

Testicular apoptotic cell death, which occurs at low levels during normal spermatogenesis, is significantly increased under diabetic conditions (Cai et al., 2000; Guneli et al., 2008; Mohasseb et al., 2011; Zhao et al., 2011). There is increasing evidence demonstrating that testicular apoptotic cell death, which may be induced by the administration of streptozotocin (STZ) in the type 1 diabetic (T1DM) rat or mouse model, occurs predominantly via activation of the mitochondrion-mediated cell death pathway (Cai et al., 2000; Koh, 2007; Sainio-Pollanen et al., 1997; Wang et al., 2014; Zhao et al., 2012; Zhao et al., 2011). These studies indicate that oxidative stress and damage play a critical role in testicular cell death in diabetic individuals.

Oxidative stress occurs in cells or tissues when the excessive generation of reactive oxygen or nitrogen species (ROS and RNS) overwhelms the endogenous antioxidant defense. Therefore, a potentially efficient approach for preventing and reducing the incidence of testicular apoptotic cell death, and consequently preventing the occurrence of infertility in diabetic males, would involve increasing the antioxidant capacity of the testis tissue. We have

previously reported that testicular apoptotic cell death in T1DM rats, induced by STZ administration, is significantly reduced by exposure to low-dose radiation (LDR) which elicits the upregulation of testicular catalase and superoxide dismutase (SOD) (Zhao et al., 2010).

LDR refers to radiation doses lower than 250 mGy of X- or γ -rays (Liu et al., 2007). In contrast to high-dose radiation, LDR induces an adaptive response or hormesis, resulting in protective effects against subsequent challenge-induced damage in vitro and in vivo (Liu et al., 2007). LDR was found to induce adaptive responses to subsequent radiation- or chemical-induced genomic damage and cell death in the testis (Liu et al., 2006; Liu et al., 2007). LDR has been extensively reported to be able to induce antioxidant production in various organs (Otsuka et al., 2006; Pathak et al., 2007; Yamaoka et al., 2002), likely via the upregulation of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) (Tsukimoto et al., 2010).

Nrf2 regulates the basal and inducible expression of genes encoding protective molecules against various oxidative stresses (Ha et al., 2006; Lee and Johnson, 2004; Lee and Surh, 2005). Nrf2, which is activated in response to a range of oxidative and electrophilic stimuli, mediates the induction of a spectrum of cytoprotective proteins such as the phase II enzymes NAD(P)H:quinone oxidoreductase (NQO-1), catalase (CAT), and SOD, as well as antioxidant proteins, e.g., heme oxygenase 1, via the antioxidant response element-dependent pathway. Nrf2, which is broadly expressed in tissues, has been recognized to play a critical role in oxidative defense in the testis (Nakamura et al., 2010; Yang et al., 2008). Nrf2 deletion or defects are known to be associated with testicular oxidative stress and spermatogenesis abnormalities in animals (Nakamura et al., 2010) and human (Yu et al., 2012).

Unlike T1DM that is predominantly characterized by lack of insulin with significant hyperglycemia, type 2 diabetes (T2DM) which accounts for at least 90% of all cases of DM is characterized by insulin resistance, shown by the hyperinsulinemia, with hyperlipidemia and mild hyperglycemia, but also shown by mild defect of insulin without hyperinsulinemia along with hyperlipidemia and significant hyperglycemia at the late stage of the disease (Andrikopoulos et al., 2008; Taylor, 1999). All of these factors also induce intracellular

energy metabolic imbalance, resulting in extra generation of ROS and/or RNS as oxidative stress, tissue chronic hypoxia, and inflammation such as NF- κ B activation in the vasculature of various organs. Whether LDR can also prevent T2DM-induced testicular apoptosis has not been addressed yet. In the present study, therefore, we investigated whether exposure to LDR stimulates testicular antioxidant production and suppresses diabetes-induced testicular apoptosis, using a T2DM rat model. In addition, we explored the potential association between LDR-upregulated expression and activity of the antioxidants CAT and SOD and the increase in Nrf2 expression and activity.

2. *Materials and methods*

2.1 *Animal models*

All animal protocols were approved by the Animal Ethics Committee of Jilin University. Male Wistar rats (Animal Center of Jilin University, Changchun, China) weighing 220-240 g were acclimated in an air-conditioned room at 22°C with a 12:12-h light-dark cycle and fed standard chow for 1 week. T2DM features two physiological defects: peripheral tissue's resistance to the action of insulin and a deficiency in insulin secretion at late stage (Andrikopoulos et al., 2008; Taylor, 1999). Accordingly an insulin-defective stage of T2DM (IDS-T2DM) rat model was created by high-fat diet (HFD) feeding containing 60 kcal% fat for 2 months to induce insulin resistance, followed by a small dose (25 mg/kg of body weight) of STZ (Sigma Aldrich, St. Louis, MO, USA) to cause mild deficiency of insulin with hyperglycemia, as described in our previous (Zhao et al., 2013) and other studies (Kusakabe et al., 2009; Luo et al., 1998; Mu et al., 2006). Control rats were fed with the control diet (CD), containing 10 kcal% fat. Both groups were given free access to water. Three days after injection of STZ, whole blood obtained from the fasting rat tail vein was used for measuring blood glucose using a SureStep Complete Blood Glucose Monitor (LifeScan, Milpitas, CA, USA). Blood glucose levels of > 250 mg/dl were considered to represent hyperglycemia. The hyperglycemic rats were considered to have late-stage T2DM with mild insulin defect, as previously described for the mouse model (Wang et al., 2014). After the onset of diabetes, rats were divided into four groups: control (Ctrl, n = 6), LDR (Ctrl/LDR, n = 6), diabetes mellitus (DM: HFD/STZ, n = 7), and DM + LDR group (DM/LDR, n = 7).

2.2 Whole-body irradiation

Rats were subjected to 25 mGy of whole-body irradiation, at a dose rate of 12.5 mGy/min, using the X-RAD 320 X-Ray Biological Irradiator (Precision X-Ray, North Branford, CT, USA) operated at 200 kVp and 10 mA with 1.0 mm aluminum and 0.5 mm copper filters. Animals were subjected to whole-body LDR under intraperitoneal anesthesia with sodium pentobarbital at 30 mg/kg in accordance with the protocols used in our previous studies on the adaptive or hormetic effects induced by LDR in the male reproductive system (Zhao et al., 2010). Animals were exposed or sham-exposed to LDR every other day for 4 weeks, with a total accumulated dose of 350 mGy. Then, animals were euthanized by sodium pentobarbital injection intraperitoneally at 150 mg/kg and their testes were harvested for histopathological and biochemical studies.

2.3 Biochemical quantification assays

Blood samples were collected from fasting rat tail vein and centrifuged at $1,200 \times g$ for 15 min in heparinized centrifuge tubes to harvest the plasma. Then, triglyceride and total cholesterol levels in the plasma were measured by triglyceride and total cholesterol quantification kits (F001-1 and F002-1, Jiancheng, Nanjing, China) respectively, according to the manufacturer's protocol. SOD activity, as well as catalase and malondialdehyde (MDA) levels in testicular tissues were assayed using the relevant quantification kits (A001-1, A007-1 and A003-1, Jiancheng, Nanjing, China). Oxidative damage was assayed by measuring the end products of lipid peroxidation, which included reactive aldehydes such as MDA and 4-hydroxynonenal (4-HNE).

2.4 Western blotting

The testicular tissues were homogenized in lysis buffer and proteins were collected by centrifuging at $12,000 \times g$ at 4°C (Cai et al., 2002). Cytoplasmic and nuclei components of testicular cells were isolated using nuclei isolation kit (NUC- 201, Sigma, MO, USA), according to the manufacturer's protocol. Western blots were performed according to our

previous studies (Cai et al., 2002). Briefly, the proteins were fractionated on 10% SDS-PAGE gels, and then were transferred to a nitrocellulose membrane. The membrane was blocked with a 5% nonfat dried milk for 1 h and incubated overnight at 4°C with the following antibodies: anti-apoptosis inducing factor (AIF), anti-Bax, anti-Bcl-2, anti-phospho-Akt (Ser473), anti-Akt, anti-Keap1, anti-phospho-GSK3 β (Ser9), anti-GSK3 β (1:1000, Cell Signaling, Beverly, MA), anti-NAD(P)H:quinone oxidoreductase 1(NQO1), anti-Fyn, anti-phospho-Fyn (Thr 12) (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), anti-3-nitrotyrosine (3-NT, 1:2000, Chemicon, Temecula, CA), anti-Nrf2 (1:1000, Abcam, Cambridge, MA), anti-protein tyrosine phosphatase-1B (PTP-1B) (1:2000, BD Biosciences, Rockville, MD), anti-4-hydroxynonenal-Michael adducts (4-HNE), and anti-Tribbles Homologue3 (TRB3) (1:1000, Calbiochem, La Jolla, CA). After removal of unbound antibodies using Tris-buffered saline (pH 7.2) containing 0.05% Tween 20, membranes were incubated with the secondary antibody for 1 h at room temperature. Antigen-antibody complexes were visualized using an enhanced chemiluminescence detection kit (Thermo Scientific, Barrington, IL). In order to determine loading, blots were stripped using stripping buffer (Sigma Aldrich, St. Louis, MO) and reprobed for β -actin as loading control of total protein. Histone was used as loading control of nuclei proteins. Quantitative densitometry was performed on the identified bands using a computer- based measurement system, as employed in previous studies (Cai et al., 2002).

2.5 Real-time qPCR

Total RNA was extracted with TRIzol reagent (Invitrogen). RNA concentration and purity were quantified using a Nanodrop ND-1000 spectrophotometer. Complementary DNA (cDNA) was synthesized from total RNA using the RNA PCR kit (Promega, Madison, WI) according to the manufacturer's protocol. Real-time qPCR was carried out in 20 μ l of reaction buffer consisting of 10 μ l of TaqMan Universal PCR Master Mix, 1 μ l of primer, and 9 μ l of cDNA. Amplification was performed in duplicate for each sample, using the ABI 7300 Real-Time PCR system. TaqMan primers for NQO1, SOD, catalase, and the β -actin control were purchased from Applied Biosystems (Cat. # 4331182, Carlsbad, CA). The

fluorescence intensity of each sample was measured to monitor amplification of the target gene. The comparative cycle time method was used to normalize the amount of target to an endogenous reference (β -actin) and relative to a calibrator ($2^{-\Delta\Delta Ct}$).

2.6 Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

Testis tissue was fixed in 10% formalin, embedded in paraffin, and sectioned at 5 μ m thickness. The slides were stained for TUNEL using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Boster, Wuhan, China), as described in previous studies (Cai et al., 2000; Zhao et al., 2010). Briefly, each slide was deparaffinized and rehydrated, and treated with proteinase K (20 mg/l) for 15 min. The endogenous peroxidase was inhibited using 3% hydrogen peroxide for 5 min, and incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and digoxigenin-11-dUTP for 1 h. The TdT reaction was carried out in a humidified chamber at 37°C. Then, 3, 3'-diaminobenzidine chromogen was added. Hematoxylin was used for counterstaining. For the negative control, TdT was omitted from the reaction mixture.

Observed under microscope, apoptotic cell death was quantitatively analyzed by counting the TUNEL-positive cells which were stained brown among 200 cells selected randomly from transections of ten seminiferous tubules by morphology from each one of the three slides for each rat. The apoptotic cells and total cells were counted only from spermatogonia, primary spermatocytes, and secondary spermatocytes identified by morphology; however, the spermatid and spermatozoa were not counted for the quantification analysis. Results have been presented as TUNEL-positive cells per 1,000 cells.

2.7 Statistical analysis

Data were collected from repeated experiments and presented as means $\pm$ S.D. One-way analysis of variance (ANOVA) was used to determine significant differences. Differences between groups were analyzed using the post hoc Tukey's test, with Origin 7.5 laboratory data analysis and graphing software. Statistical significance was considered as $P < 0.05$.

Results

3.1 T2DM model and effects of LDR on T2DM magnification and testicular cell death

T2DM was induced by feeding rats HFD for 2 months, followed by the administration of a single dose of STZ (25 mg/kg), as illustrated in Fig. 1A. After the onset of hyperglycemia, diabetic and age-matched control rats were treated with or without LDR at 25 mGy for 4 weeks every other day. As shown in Fig. 1B, at the end of the treatment period, body weights of the rats in the diabetic group were significantly increased regardless of LDR exposure. Repetitive exposure to LDR did not affect body weight in either control or diabetic rats. Diabetes was associated with significantly increased blood glucose levels (Fig. 1C, fasting), plasma total cholesterol levels (Fig. 1D), and triglyceride (Fig. 1E) levels. Exposure to LDR did not affect these variables in the normal and diabetes groups. Diabetes was associated with a significant decrease in the testis weight/tibia length ratio (Fig. 1F); however, this was not affected by LDR exposure.

Testicular apoptosis was examined by TUNEL staining for TUNEL-positive cells (A) and by western blotting assay for the Bax/Bcl-2 ratio (B) and AIF (C). Although LDR exposure did not affect TUNEL-positive cells, it was found to reduce the Bax/Bcl-2 expression ratio and AIF expression levels. Diabetes was associated with a significant increase in testicular apoptotic cell death, as shown by TUNEL staining, as well as in the Bax/Bcl2 expression ratio and AIF levels. LDR exposure was found to significantly, but not completely, prevent the diabetes-induced apoptotic effect. These results suggest that T2DM is associated with the induction of the mitochondrial AIF-dependent apoptotic cell death pathway, which may be significantly reduced by LDR exposure.

3.2 Effect of LDR on T2DM-induced testicular oxidative damage

We have previously demonstrated that T1DM-induced testicular apoptotic cell death occurs mainly due to testicular oxidative stress and damage (Zhao et al., 2011). Here, we additionally demonstrate significant increases in testicular 3-NT (Fig. 3A) and 4-HNE (Fig. 3B) accumulation as indices of protein nitration and lipid peroxidation, respectively, by western blotting assay. The increased lipid peroxidation was further confirmed by chemical

assay of reactive aldehydes such as MDA, which represent the end products of lipid peroxidation (Fig. 3C). LDR exposure significantly reduced testicular 3-NT accumulation in the control group and prevented diabetes-induced testicular accumulation of both 3-NT as well as end products of lipid peroxidation (4-HNE and MDA).

3.3 Possible mechanisms by which LDR prevents testicular oxidative stress and damage

The expression of Nrf2 was detected by western blotting assay (Fig. 4A). The ratio of nuclear Nrf2 to cytoplasmic Nrf2 was utilized as an index of Nrf2 transcription. Accordingly, the expression of Nrf2-downstream target genes (NQO1, SOD, and CAT) were evaluated by real-time PCR (Fig. 4B). Additionally, biochemical assays were performed for SOD activity (Fig. 4C) and catalase content (Fig. 4D). The ratio of nuclear Nrf2 to cytosolic Nrf2 was increased in both DM and LDR groups, and further increased in DM/LDR group, suggesting that the transcription activity of Nrf2 was increased. suggesting that Nrf2 transcription levels were increased In line with this notion, mRNA levels of NQO1, SOD, and CAT were significantly decreased in the DM group and DM/LDR group, and further decreased in DM group, but significantly increased in the LDR group (Fig. 4B). Consistent with the mRNA profiles, SOD activity and catalase content were also significantly decreased in DM group, significantly increased in LDR group, and the changes in DM/LDR group were between DM and LDR group. (Figs. 4C and D). This suggests that LDR may stimulate the production of Nrf2-mediated downstream antioxidants to protect against diabetes-induced damage, as shown in Fig. 3.

In order to investigate the mechanisms by which LDR stimulates Nrf2 function under diabetic conditions, the testicular expression of Keap1 was examined by western blot assay (Fig. 5A). A slight (statistically different) decrease in the DM and LDR groups and synergistic decrease in the DM/LDR group were observed. Due to the principle mechanism by which ionizing radiation affects tissue by ionizing water generating ROS, this suggests that LDR may generate small amount of ROS to oxidize Keap1, leading to ubiquitin-mediated degradation.

Furthermore, the expression of phosphorylated and total Akt (Fig. 5B), GSK-3 β (Fig. 5C), and Fyn (Fig. 5D) were measured by western blotting assay. Diabetes was associated with

significant reductions in Akt and GSK-3 β phosphorylation, and an increase in nuclear Fyn accumulation, which suggests that nuclear Fyn plays a role in exporting nuclear Nrf2, resulting in the inhibition of Nrf2 function, In parallel with the results shown in Fig. 4, exposure to LDR increased the phosphorylation levels of Akt and GSK-3 β in non-diabetic rats. However, phosphorylation levels of Akt and GSK-3 β were maintained at the same level in diabetic rats (Figs. 5B and C). Exposure to LDR additionally decreased nuclear Fyn accumulation under both non-diabetic and diabetic conditions (Fig. 5D).

Since we have demonstrated the increased expression of Akt negative regulators, TRB3 and PTP1B, in the testis of mice with T1DM (Zhao et al., 2012), The expression of TRB3 (Fig. 6A) and PTP1B (Fig. 6B), two typical Akt negative regulators, was examined by western blotting assays. The results showed that diabetes significantly increased the expression of TRB3 and PTP1B. Exposure to LDR did not elicit significant effects in the non-diabetic group; however, a significant, albeit partial, reduction was observed in the expression of both TRB3 and PTP1B in the diabetic group.

Discussion

The present study explored the effects of LDR on T2DM-induced testicular cell death, and the mechanisms underlied these effects. Consistent with recent findings in the HFD/STZ-induced T2DM mouse model, HFD/STZ-induced T2DM diabetes in the rat model was found to be significantly associated with a decrease in testicular weight and an increase in testicular cell death. We demonstrated that T2DM-induced testicular cell death is significantly associated with an increase in mitochondrial Bax expression and AIF expression, but not caspase-3 cleavage. In addition, T2DM caused significant increase in testicular lipid peroxidation and protein nitration, along with a modest degree of Nrf2 upregulation. To our knowledge, this study is the first to demonstrate that LDR exposure significantly, albeit partially, prevents the above pathogenic changes induced by T2DM in the testes of rats. Furthermore, the protective effect exerted by LDR exposure against T2DM-induced testicular apoptosis was associated with Nrf2 activation. It is likely that this effect occurs due to the suppression of the negative regulators of Akt (TRB3 and PTP1B), which in turn release their inhibition of Akt. Activated Akt then phosphorylates GSK-3 β to reduce its activation

(phosphorylation) of Fyn. The inactivated Fyn stays in cytosol, indirectly stabilizing Nrf2 function due to the reduction of its exporting Nrf2 from nuclear to cytosol where Nrf2 would be degraded, as illustrated in Fig. 6B.

Infertility is a common complication observed in men with diabetes. The main contributor to diabetes- induced infertility is the loss of germ cells due to increased apoptotic cell death (Cai et al., 2000; Dinulovic and Radonjic, 1990; Koh, 2007; Sainio-Pollanen et al., 1997). Several studies in T1DM animal models, including our own (Zhao et al., 2010; Zhao et al., 2012), have indicated that male germ cell death is predominantly mediated by the mitochondrial cell death pathway. However, obesity- or T2DM-induced testicular effects have not received much attention. To our knowledge, there have been only few reports indicating that type 2 diabetes induces testicular cell death in animal models (Wang et al. 2014) and human patients with diabetes (Roessner et al., 2012).

An early human study of semen samples collected from 18 healthy fertile donors and 27 donors with T1DM (n = 13) or T2DM (n =14) assessed apoptosis based on indicators such as disrupted transmembrane mitochondrial potential, activation of caspase 3, and DNA fragmentation, using flow cytometry. It was found that the ejaculate of diabetic men contained significantly higher concentrations of spermatozoa with disrupted transmembrane mitochondrial potential, activated caspase 3, ROS, and fragmented DNA compared with the semen of healthy fertile donors. This effect is more pronounced in men with T2DM. The present study shows, for the first time, that apoptosis signaling is significantly increased in sperm from males with T1DM and T2DM (Roessner et al., 2012). Using the mouse model, we demonstrated for the first time that testicular apoptosis significantly upregulates mitochondrial pathways, as indicated by the increased ratio of Bax/Bcl2 expression and cleavage of caspase-3 as well as the significantly increased levels of testicular oxidative damage and inflammation.

In support of the above mouse model, we further demonstrate that in HFD/STZ-induced T2DM rat model, testicular apoptosis (Fig. 2A) is also significantly elevated along with significant increases in oxidative stress and damage levels (Fig. 3), Bax/Bcl2 ratios, and AIF expression (Fig. 2B,C) in the current study. The above two studies in mouse and rat models of diabetes suggest that T2DM, like T1DM, is associated with a significant increase in

testicular cell death. These findings should explain why patients with T2DM show an increase in the levels of markers of apoptotic death in their semen (Roessner et al., 2012).

The most interesting finding of the present study is the preventive effect of LDR exposure on T2DM-induced testicular apoptosis and associated pathological changes. We have previously reported the distinct effect of LDR from that of higher doses of radiation, in that LDR is able to induce adaptive responses as reflected by the resistance to subsequent challenge-induced toxic effects (Liu et al., 2006; Liu et al., 2007; Otsuka et al., 2006; Pathak et al., 2007; Tsukimoto et al., 2010; Yamaoka et al., 2002). We have additionally demonstrated that diabetic mice exposed to repetitive LDR at 25 mGy showed significant prevention of diabetes-induced renal and cardiac damage in T1DM (Zhang et al., 2009; Zhang et al., 2011) and T2DM models (Shao et al., 2014). In the T1DM rat model, we have demonstrated the preventive effect of LDR on diabetes-induced testicular cell death via upregulation of catalase and SOD (Zhao et al., 2010). Consistent with the previous study, we present evidence that LDR also protects the testis from T2DM-induced testicular apoptotic cell death and elicits the upregulation of SOD activity and CAT levels (Figs. 4C and D). Furthermore, we demonstrate that LDR-increased CAT activity and SOD levels are associated with the upregulation of Nrf2 function, as evidenced by increased nuclear mRNA levels of Nrf2 and Nrf2-downstream genes NQO-1, CAT, and SOD (Fig. 4B). It is known that CAT and SOD are both Nrf2 downstream genes. Therefore, we assumed that LDR stimulates Nrf2 function to upregulate the production of numerous antioxidants, including NQO-1, CAT, and SOD, resulting in the prevention of diabetes-induced oxidative damage and associated apoptotic cell death.

Another novel finding of this study is that LDR-induced upregulation of Nrf2-dependent antioxidant function likely occurs via the suppression of Akt negative regulators TRB3 and TPT1B, which prevents the inhibition of Akt phosphorylation. The activated (phosphorylated) Akt phosphorylates (inactivates) GSK-3 β , resulting in the translocation of Fyn into the nucleus, where phosphorylated Fyn exports Nrf2 from nuclei to cytosol, resulting in ubiquitin-mediated degradation of Nrf2, as illustrated in Fig. 5C. Although there have been no reports regarding the suppressive effect of LDR exposure on TRB3 and TPT1B to date, several studies have indicated the stimulatory effect of LDR exposure on Akt and Nrf2 (Kim

et al., 2007; Xing et al., 2012), which results in the upregulation of several cell survival and antioxidant signaling pathways. It has been reported that the inactivation of PTP1B occurs due to low concentrations of oxygen generated by photosensitization (von Montfort et al., 2006). We assume that LDR generates small amounts of ROS that may in turn inactivate PTP1B, as observed in the present study (Fig. 5B). In addition, it is known that Keap1, which is enriched in cysteine, promotes Nrf2 ubiquitin-mediated degeneration. Several compounds, such as sulforaphane, are able to oxidize Keap1 to release Nrf2, thereby stabilizing Nrf2 function (Ha et al., 2006; Lee and Johnson, 2004; Lee and Surh, 2005). We have demonstrated that Nrf2 is functionally activated by sulforaphane to protect the testis from diabetes-induced effects (Wang et al., 2014). Therefore, we assume that LDR may also generate small amounts of ROS to oxidize Keap1, thereby releasing and stabilizing Nrf2, as illustrated in Fig. 6C.

A potential limitation may be noticed that although LDR can stimulate a testicular protection against from diabetes, it remains unclear whether LDR can be potentially applied to clinics for diabetic patients. To date whether exposure to LDR, particularly at doses less than 100 mGy, really increases cancer risk remains a big debate (Scott et al., 2012; Socol and Welsh, 2015). Our body is a complicate system in which anticancer systems and carcinogenesis is always a dynamic balance. Although LDR may cause small amount of DNA damage, it also stimulates multiple defense systems to prevent the carcinogenesis (Cheda et al., 2004; Otsuka et al., 2006). In a line with this notion, in animal studies, exposure of normal or tumor-bearing mice to 25 – 75 mGy LDR significantly prevented the tumor growth and metastasis (Cheda et al., 2004; Nowosielska et al., 2011; Scott et al., 2012). Human data also did not show the significant increase in cancers among population exposed to such LDR (Laborde-Casterot et al., 2014; Lehrer and Rosenzweig, 2015; Wakeford, 2014). In obese or type 2 diabetic models, there might be additional cancer risk since insulin resistance and elevated levels of insulin can displace insulin growth factor (IGFs) from IGF binding proteins resulting in increased levels of free IGFs that may constitutively promote tumor growth and cancer progression (Leone et al., 2014). In the other side, however, LDR also stimulates Akt signaling in multiple organs including testis (Fig. 5) and kidney (Xing et al., 2012), which may enhance peripheral tissue insulin sensitivity, resulting in decreased

circulating insulin level. Therefore, whether the preventive dose of LDR used here will increase cancer risk needs to be systemically investigated. The direct goal of the present study is to define whether up-regulation of testicular antioxidant system such as Nrf2-mediated anti-oxidative defense system is able to protect the testis from type 2 diabetes via a non-invasive approach to avoid blood-testis barrier, which may provide an alternative approach to investigate the potential preventive strategy potentially for diabetic patients in the future.

In summary, here we demonstrated for the first time that repetitive exposure to LDR at 25 mGy protect T2DM-induced germ cell apoptotic death along with the increase in testicular Nrf2 expression and function. However, whether Nrf2 up-regulation and activation is the pivotal role in the testicular protection from diabetes remains further confirmed with *Nrf2* gene knock mice. In addition, although we demonstrated the up-regulation of Nrf2 expression and function was associated with LDR suppression of Akt negative regulators and also Keap1 expression, whether these two associated phenomenon are the direct mechanism responsible for LDR's stimulation of Nrf2 remain further defined.

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Conflicts of interest

No conflicts of interest. All authors takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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Figure captions

Fig.1. General effects of LDR on T2DM rats. T2DM was induced in rats by 2 months HFD feeding followed by administration of a small dose of STZ at 25 mg/kg. After the onset of hyperglycemia, diabetic and age-matched control rats were treated with or without LDR at 25 mGy for 4 weeks every other day (A). At the end of the treatment period, body weights (B), blood glucose levels (C), plasma total cholesterol levels (D), plasma triglyceride levels (E), and the testis weight/tibia length ratios (F) were determined. Data are presented as mean $\pm$ SD (n = 6 at least in each group). DM: diabetes. LDR: low dose radiation. *, P < 0.05 vs. control group; #, P < 0.05 vs. DM.

Fig. 2. Effects of LDR on T2DM-induced testicular apoptosis. T2DM and control rats were subjected to the same treatment, as described in Fig. 1. Testicular apoptotic cell death was examined by TUNEL staining. TUNEL-positive cells were quantitatively analyzed (A). The expression of Bax and Bcl-2 was detected by western blotting assay (B), for which the ratio of Bax/Bcl-2 was presented. Testicular apoptosis expression was examined by western blotting assay for the expression of AIF (C). Data are presented as means $\pm$ SD (n= 6 at least, in each group). DM: diabetes. LDR: low dose radiation. *, P < 0.05 vs. control group; #, P < 0.05 vs. DM.

Fig. 3. Effects of LDR on T2DM-induced testicular oxidative damage. T2DM and control rats were subjected to the same treatment, as described in Fig. 1. Testicular oxidative damage was examined by western blotting assay for the expression of 3-NT as an index of protein nitration (A) and 4-HNE as an index of lipid peroxidation (B). Activity of MDA was confirmed by chemical quantification assay (C). Data are presented as means $\pm$ SD (n = 6 at least, in each group). DM: diabetes. LDR: low dose radiation. *, P < 0.05 vs. control group; #, P < 0.05 vs. DM.

Fig. 4. Effects of LDR on testicular Nrf2 expression and function. T2DM and control rats were subjected to the same treatment, as described in Fig. 1. The expression of Nrf2 was detected by western blotting assay (A), for which the ratio of nuclear Nrf2/cytosolic Nrf2 was presented. mRNA levels of NQO1, SOD, and CAT were

detected by real-time qPCR (B). Activity of SOD (C) and CAT levels (D) were assayed using the corresponding quantification kits. Data are presented as means $\pm$ SD (n = 6 at least, in each group). DM: diabetes. LDR: low dose radiation. *, P < 0.05 vs. control group; #, P < 0.05 vs. DM.

Fig. 5. Effects of LDR on testicular Akt expression and function. T2DM and control rats were subjected to the same treatment, as described in Fig. 1. Testicular expression of Keap1 (A), phosphorylated and total Akt (B), GSK-3 β (C), and Fyn (D) was measured by western blotting assay. Data are presented as means $\pm$ SD (n = 6 at least, in each group). DM: diabetes. LDR: low dose radiation. *, P < 0.05 vs. control group; #, P < 0.05 vs. DM.

Fig. 6. Effects of LDR on negative regulators of testicular Akt expression. T2DM and control rats were subjected to the same treatment, as described in Fig. 1. The expression levels of TRB3 and PTP1B (negative regulators of Akt) (A, B) were measured by western blotting assay. Data are presented as means $\pm$ SD (n = 6 at least, in each group). DM: diabetes. LDR: low dose radiation. *, P < 0.05 vs. control group; #, P < 0.05 vs. DM. The working hypothesis for the mechanisms underlying LDR-induced attenuation of testicular apoptosis in T2DM are illustrated in panel C.

A Procedure

B

C

D

E

F

Fig. 1

A **B** **C** **Fig. 2**

Fig. 3

Fig. 4

Fig. 5

Fig. 6