Antioxidant protects blood-testis barrier against synchrotron radiation X-ray-induced disruption

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Abbreviations: AJC, apical junctional complex; BTB, blood-testis barrier; BCA, bicinchoninic acid; DAPI, 4,6-diamidino-2-phenylindole; DSB, double-strand DNA breaks; ECL, enhanced chemiluminescence; GSH, γ-glutamylcysteinylglycine; i.p, intraperitoneal; MAGUK, membrane-associated guanylate kinases homologs; NAC, N-Acetyl-L-Cysteine; PBS, phosphate-buffered saline; RT, room temperature; SR, Synchrotron radiation; TJ, tight junction

Synchrotron radiation (SR) X-ray has wide biomedical applications including high resolution imaging and brain tumor therapy due to its special properties of high coherence, monochromaticity and high intensity. However, its interaction with biological tissues remains poorly understood. In this study, we used the rat testis as a model to investigate how SR X-ray would induce tissue responses, especially the blood-testis barrier (BTB) because BTB dynamics are critical for spermatogenesis. We irradiated the male gonad with increasing doses of SR X-ray and obtained the testicles 1, 10 and 20 d after the exposures. The testicle weight and seminiferous tubule diameter reduced in a dose- and time-dependent manner. Cryosections of testes were stained with tight junction (TJ) component proteins such as occludin, claudin-11, JAM-A and ZO-1. Morphologically, increasing doses of SR X-ray consistently induced developing germ cell sloughing from the seminiferous tubules, accompanied by shrinkage of the tubules. Interestingly, TJ constituent proteins appeared to be induced by the increasing doses of SR X-ray. Up to 20 d after SR X-ray irradiation, there also appeared to be time-dependent changes on the steady-state level of these protein exhibiting differential patterns at 20-day after exposure, with JAM-A/claudin-11 still being up-regulated whereas occludin/ZO-1 being down-regulated. More importantly, the BTB damage induced by 40 Gy of SR X-ray could be significantly attenuated by antioxidant N-Acetyl-L-Cysteine (NAC) at a dose of 125 mg/kg. Taken together, our studies characterized the changes of TJ component proteins after SR X-ray irradiation, illustrating the possible protective effects of antioxidant NAC to BTB integrity.

Introduction

Synchrotron Radiation (SR) X-ray is a coherent, monochromatic, collimated light source with very high photon flux. It represents an immense leap in performance over conventional x-ray sources, presenting an immense potential in biomedical imaging and tumor therapy.1–4 However, there are still multiple questions underlying the interactions between SR X-ray and biological tissues. For example, it is known that conventional X-ray induces gonad damages, especially in the primate testis as it is a highly sensitive target for irradiation due to the rapidly dividing germ cells.4 Thus, it is important and urgent to determine the safe doses of SR X-ray, and to search for the strategies to reduce the detrimental effects on normal tissues.

The blood-testis barrier (BTB) is a physical barrier between adjacent Sertoli cells in the testicles of the mammalian testes, which creates a unique microenvironment for the development and maturation of meiotic and postmeiotic germ cells in seminiferous tubules.5,6 The BTB is a complex structure between adjacent Sertoli cells, and is composed of not only tight junction proteins such as occludin, JAM-A, claudin-11 and ZO-1, but also anchoring junction proteins like N-cadherin and catenins.6 The BTB divides the seminiferous epithelium into basal and adluminal compartments that becomes critical for spermatogenesis and germ cell development, partly by preventing passage of cytotoxic agents into the seminiferous tubules.6 While this process can be regulated by many factors,7,8 it!remains to be investigated if SR X-ray will affect the BTB function. Recently we have demonstrated that NAD+, an important metabolite that is involved in energy metabolism, mitochondrial functions and others could attenuate SR X-ray-induced DNA damage and structural alterations in the testis.9 However whether such alterations involved blood-testis barrier restructuring remain to be addressed. Herein we use the rat testicle as a model to explore the effect of different doses of SR X-ray on BTB dynamics.
N-Acetyl-L-Cysteine (NAC) is a nontoxic molecule that enters cells readily and serves as an antioxidant and a precursor for glutathione (γ-glutamylcysteinylglycine, GSH). It blocks the activation of NF-κB that is involved in stress-induced, immune, or inflammatory responses by reducing inflammation of tissues and cells. In this study we also examined if NAC administration could attenuate SR X-ray-induced injury to the testes in reference to BTB damage. We focus on the tight junction proteins alone in this report.

**Results**

**SR X-ray irradiation results in the loss of testicle weight and shrinkage of seminiferous tubule diameters**

We first examined the gross changes of rat testicles after SR X-ray irradiation. Increasing doses of SR caused the sloughing of germ cells from the seminiferous epithelium, leading to reduction of testis weight and shrinkage of seminiferous tubules. In the present study, we found that 40 Gy SR X-ray could significantly decrease the weight of the testes as early as 1 day after exposure (Fig. 1 A). Our data also indicated that lower doses at 1.3 Gy and 4 Gy caused testicle weight reduction at 20 d after exposure (Fig. 1 A). Correspondingly, the diameter of seminiferous tubules apparently shrunk at 10 d and 20 d after 4 Gy radiation (Fig. 1 B), and the dose at 40 Gy caused more profound reduction (Fig. 1 B). At this highest dose, tubule diameter was affected as early as 1 day after exposure. These physiological state alterations strongly indicated the underlying cellular and molecular changes in the testis after SR X-ray exposure.

High doses of SR X-ray disrupt the blood-testis barrier function by altering the level of tight junction proteins

The role of tight junction proteins on spermatogenesis, such as via a restriction of paracellular flow of biomolecules, creation of an immunological barrier and conferring cell polarity in the seminiferous epithelium, has been illustrated in many studies. The integral membrane proteins JAM-A, claudin-11 and occludin and the adaptor protein ZO-1 of the tight junction are known to be critical components of the BTB. We first examined whether SR X-ray had an effect on the BTB integrity by using immunofluorescence microscopy to examine the distribution of these proteins at the BTB, to be followed by changes of protein levels by Western blot, in the treatment vs. the control group.

In the control animal group, seminiferous tubules arranged orderly and the sizes of the tubule lumens were mostly uniform. JAM-A signal had no apparent change 1 day after exposure of the testis to SR X-ray at doses of 0.5 Gy, 1.3 Gy, 4 Gy or 40 Gy compared to the control (Fig. 2 A, left panel). However, at 10 or 20 day after exposures, especially at 20 day, seminiferous tubules became disorganized and shrunken in sizes considerably with JAM-A signal apparently became upregulated at the radiation doses of 4 Gy and 40 Gy. Detailed examination of the seminiferous tubules identified dislodging of germ cells from the epithelium, also illustrating structural damage after radiation. Moreover, the increased JAM-A appeared to localize to the basal compartment, but also diffuse to the adluminal compartment, especially for higher doses at longer time points (Fig. 2A, middle and right panel). In contrast, at the doses of 0.5 Gy and 1.3 Gy, the JAM-A signal had little changes, and the variation of the pattern and size of seminiferous tubules were minimal (Fig. 2 A, middle and right panel). It was not surprising to find that JAM-A levels appeared up-regulated upon toxicant challenge, plausibly as a defensive mechanism for barrier protection.

To corroborate these results, Western blot analysis of lysates also showed that at 4 Gy and 40 Gy SR X-ray radiation 20 d after exposure, but not at 0.5 Gy or 1.3 Gy, the protein level of JAM-A in the testes was significantly increased (Fig. 2 B and C). Contrarily, at 1 day after SR X-ray treatment, JAM-A levels appeared unaltered even at higher doses of 40 Gy (Fig. 2 B and C).

Similarly, we examined changes in the distribution of claudin-11, occludin, and ZO-1 by immunofluorescence staining, and quantified the protein levels of claudin-11 and occludin by Western blot (Fig. 3). JAM-A, claudin-11 appeared to be sensitive to the SR X-ray radiation based on results of Western blot data (Fig. 3 B and C). At 20 d after the SR X-ray radiation, 1.3 Gy SR X-ray significantly increased claudin-11 protein level compared to control group, and the protein level was even higher after 4 Gy and 40 Gy SR X-ray radiation (Fig. 3 B and C). On the other hand, changes of occludin as measured by immunofluorescence microscopy appeared to respond faster than JAM-A or claudin-11, since an up-regulation of detectable signals was observed at 10 d after radiation (Fig. 4 A). It appeared that at 1 day and 10 days, occludin displayed the same trend as...
JAM-A and claudin-11 (Fig. 4 A, left and middle panel, B and C). At 20 d after the SR X-ray exposure (4 Gy or 40 Gy), occludin levels plummeted dramatically compared with control (Fig. 4 A, right panel, B and C). Cell junction adaptor protein ZO-1 showed very similar changes as occludin, in which an obvious increase could be observed at 10 days, followed by a marked reduction in the fluorescence immunostaining at 20 d after exposing to 40 Gy SR X-ray radiation (Fig. 5).

NAC administration attenuated SR X-ray radiation-induced BTB disruption

The testis appears to be a sensitive model to evaluate SR X-ray induced tissue damage by screening the effects of radiation protective agents. Herein we used a common antioxidant NAC to examine its protective effect on the BTB integrity after SR X-ray. We measured IgG protein extravasation by immunofluorescence staining as an indicator of the BTB integrity. Immunofluorescence microscopic examination of the rat IgG revealed that a tremendous amount of IgG permeated into the seminiferous tubules 1 day after 40 Gy SR exposure, compared with the control group in which no green fluorescence was detected inside the tubules (Fig. 6 A, top 2 panels). Administration of NAC at doses of 125 mg/kg one hour before irradiation could significantly reduce IgG leakage across the BTB (Fig. 6 A, bottom panel). However, the lower dose of NAC at 25 mg/kg offered no statistically significant BTB protection against SR X-ray (Fig. 6B), but certain seminiferous tubules did show very little intratubular immunofluorescence signals (Fig. 6 A, the third panel). We then examined immunofluorescence patterns of tight junction component proteins in these samples. 40 Gy SR irradiation caused germ cell sloughing as evidenced by DAPI staining in a number of seminiferous tubules that was coupled with diffused occludin immunofluorescence (Fig. 7). Treatment of NAC at a higher dose (125 mg/kg) could markedly reverse such pattern with few tubules presenting germ cell loss (Fig. 7). Together these results demonstrated that antioxidant NAC could effectively attenuate the SR X-ray induced BTB disruption as measured by the marked reduction of the IgG leakage into the seminiferous tubules.

Discussion

Biomedical applications of SR X-ray, in particular its use on live subjects require a better understanding of its interaction with
biological tissues. In the present study we showed that SR X-ray could induce structural changes of the seminiferous tubules and molecular alterations of the BTB based on analyses of TJ-associated proteins. Lower doses at 0.5 or 1.3 Gy induced little structural or molecular changes both at 1 day and 20 d after exposure. For higher doses at 4 Gy and 40 Gy, BTB constituent proteins JAM-A, claudin-11, and occludin displayed no apparent changes in the protein levels 1 day after exposure but functional barrier integrity could be compromised even with very little morphological changes of the seminiferous tubules. Whereas at 10 and 20 days, both the TJ protein levels and structures of seminiferous tubules showed drastic changes at the radiation doses of 4 Gy and 40 Gy. Apparently the upregulation of JAM-A and claudin-11 signals at 10 or 20 d and occludin signal at 10 d after exposure to 40 Gy SR X-ray could be the result of tubule shrinkage and enrichment of supporting cells. Such changes suggest a tissue protective response in the BTB, which is critical for maintaining spermatogenesis and germ cell development. Moreover, we provided evidence that SR X-ray-induced damage of normal gonad tissue damage could be significantly attenuated by NAC administration, which may be useful for safe use of SR X-ray in the clinical practice. This was the first study to provide the characterization of changes of TJ constituent proteins in SR X-ray-induced testicular damage.

Male gonad is a very sensitive organ to radiation damage including that from the SR X-ray. The BTB is an important ultrastructure in the seminiferous tubules of the mammalian testis. TJ proteins, such as JAM-A, claudin-11, occludin, ZO-1, and N-cadherin, play a key role in keeping the integrity of the structure and the stability of the internal environment. JAM-A is a transmembrane, TJ-associated Ig superfamily member that regulates epithelial barrier function, cell migration, and proliferation. Many factors which affect junctional integrity could alter JAM-A expression and localization, such as cytokines, tissue damage, X-ray (Fig. 2) and others. The claudins fulfill the task of establishing barrier properties but share no homology to the 4 transmembrane domains of occludin. The functions of claudins in regulating barrier integrity of distinct epithelial tissues were revealed by specific claudin knockout mice. For example, Nittaet...
demonstrated claudin 5 knockout mice die shortly after birth with defects in blood-brain barrier, whereas claudin 11 knockout mice have male sterility attributed to the lack of TJ between Sertoli cells of the testes. Occludin was the first TJ integral transmembrane molecule discovered, and occludin-deficient mice developed chronic inflammation, calcifications in the brain and around brain vessels, hyperplasia of the gastric epithelium, postnatal growth retardation, testicular atrophy and abnormalities in sexual behavior. ZO-1 is a scaffold TJ protein that belongs to the membrane-associated guanylate kinases homologs (MAGUK) protein family which associates directly with other integral and adaptor proteins of the TJ or gap junctions, and is believed to organize these proteins within the apical junctional complex. Oxidative stress like hypoxia, vitamin deficiencies, diabetes, inflammation and so forth can alter ZO-1 protein expression. ZO-1 forms a link between the cortical cytoskeleton and the transmembrane TJ proteins such as occludin, claudin, or JAM. Inflammatory cytokines could enhance the permeability of endothelial and epithelial barriers by inducing clathrin-dependent internalization of apical junctional complex (AJC) proteins that include JAM-A, occludin, and N-cadherin. It cues that enhanced barriers may be protective against chronic inflammation and damage by up-regulating TJ protein levels. Thus in our study, up-regulation the level of each TJ protein after SR X-ray at 10 d may be a compensation mechanism to the radiation damage. Our current study found that the antioxidant NAC could attenuate SR X-ray-induced BTB damage 1 day after high dose irradiation. In addition, we have previously showed that NAC could attenuate SR X-ray-induced double-strand DNA breaks (DSB) one day after SR X-ray irradiation. It has also been observed that SR X-ray irradiation of rodent testes produced poly (ADP-ribose) polymerase activation, which was also preventable by NAC administration. Taken together, these results have strongly indicated a critical role of oxidative stress in the testicular tissue injury induced by high doses of SR X-ray, and suggested that administration of antioxidants served as an applicable strategy to decrease SR X-ray-induced injury of normal tissues.

There are some limitations in this study. Regarding the BTB function, we focused on an animal model that received high-dose of SR X-ray but within a shorter time frame. Studies comprised of longer time points, or of lower doses with extended observation.

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**Figure 4. High doses of SR X-ray induced changes of tight junction protein occludin** Testicular samples were collected 1 day, 10 d and 20 d after SR X-ray irradiation. (A) Immunofluorescence staining for occludin (green), with DAPI staining for nuclei. Scale bar = 100 μm. (B) Representative immunoblots for occludin levels in rat testes lysates. The same blot was also probed with actin as a loading control. (C) Quantifications of the immunoblot in each group. Data are mean ± SEM, n = 6–8 in each group. **P < 0.01 (compared with control).
window should be included in a future study to provide a better characterization of the BTB dynamics after SR X-ray. Another limitation is that we did not evaluate the spermatogenic capacity after SR X-ray, especially at lower doses we used in this study such as reversibility, sperm counts and others. Further study is warranted to determine the dose of SR X-ray at which the male gonad will retain its spermatogenic capacity to repopulate the seminiferous tubule. It will also be of great interest to study the long-term impact of SR X-ray on gene mutation, chromosomal aberrations or the decline in sperm quality and pertinent functional parameters such as sperm capacitation.

**Methods and Materials**

**Animals**

Male Spraque-Dawley rats (~200g) were maintained under a 12:12-h light/dark cycle with free access to food and water. All animal procedures were approved by the Institutional Animal Care and Use Committee, Shanghai Jiao Tong University (Shanghai, China).

Before exposures of male gonads to SR X-ray, rats were anesthetized by intraperitoneal (i.p.) injection with 10% chloral hydrate. Then rats were randomly divided into groups, and the testicles were irradiated (all other body parts were lead shielded) with increasing doses (0 Gy, receiving all procedures except radiation; 0.5 Gy; 1.3 Gy; 4 Gy and 40 Gy) of SR X-ray at BL13W1 of Shanghai Synchrotron Radiation Facility (SSRF), China. Dosage calculation and detailed procedures followed previous reports.9

For antioxidant treatment experiment, rats were randomly divided into NAC (at 25 or 125 mg/kg) and vehicle (same volume of normal saline as NAC) groups, and 1 h after intravenously administration of each agent, their gonads were exposed to 40 Gy of SR X-ray. Thereafter rats were housed until the time point of investigation.

**Calculations of radiation doses**

We calculated the radiation doses based on the air kerma method as previously described.41 In brief, the photon flux of SR X-ray was determined through employing an ionization chamber.
by measuring the ionized electron currents. Thereafter the photon flux was used to calculate the air kerma at the entrance of the tissues. The average radiation dose of the samples was then converted from air kerma.

Sample collection
At different time points after SR X-ray exposures, rats were anesthetized with i.p. of 10% chloral hydrate and the testes were removed, weighed and then snap frozen in liquid nitrogen until further analysis. Animals were terminated via cervical dislocation.

Immunofluorescence staining
Cryosections of testes were obtained by a Leica Cryostat and mounted onto poly-L-lysine coated slides and stained with specific antibodies to blood-testis barrier constituent proteins occludin, claudin-11, JAM-A and ZO-1. Briefly, cryosections were fixed in 4% paraformaldehyde for 15 min at room temperature (RT), then washed 3 times in PBS, and the slices were blocked in 10% normal goat serum in PBS for 30 min at RT. Cryosections were incubated with rabbit anti-claudin-11/JAM-A/ZO-1 antibodies (1:200, Life Technologies) and rabbit anti-occludin antibodies (1:250, Life Technologies) in 1% of the blocking serum at RT for 4 h or at 4°C overnight. After three washes with PBS, the slides were incubated with Alexa Fluor 488 goat anti-rabbit antibody (1:200, Life Technologies) containing with 1% normal goat serum at RT for 1 h in darkness. Sections were then stained in 0.2% DAPI solution (Beyotime, China) for 5 min. After washing with PBS, slides were mounted with antifade mounting medium (Beyotime, China) and viewed under a Leica upright microscope (Leica DM2500, Germany). For IgG detection in the seminiferous epithelium to indicate the integrity of BTB, these testicular sections were incubated with goat anti-rat IgG conjugated with biotin (1:200, Life Technologies), and visualized by adding with avidin-Alexa Fluor 488.

Western blotting
The testicular samples were sheared, briefly ultrasonically processed, and lysed in ice-cold RIPA buffer (Millipore, Figure 6. NAC inhibited 40 Gy SR X-ray radiation induced blood-testis barrier leakage assayed by detection IgG inside seminiferous tubules. Testicular samples were collected 1 day after SR X-ray irradiation (40 Gy with or without NAC pretreatment). (A) Immunofluorescence staining for IgG (green), with DAPI staining for nuclei. Scale bar=200 μm on the left and 100 μm on the right. n=6–8 in each group. (B) Quantifications of the damage degree of seminiferous tubules – in each group. Tubules were grouped according to the level of damage (normal, low and high) and data were analyzed by χ² statistic. P < 0.05 was considered statistically significant. **P < 0.005.
Temecula, CA) containing protease inhibitor cocktail (Roche) and PMSF, and then centrifuged at 12,000 rpm for 20 min at 4°C. A bicinchoninic acid (BCA) assay kit (Thermo Scientific, Rockford, IL) was used for total proteins quantification. Sample incubated with goat anti-rabbit polyclonal HRP-conjugated antibody solutions (1:500) (rabbit anti-occludin/claudin-11/JAM-A/ZO-1 antibodies, Life Technologies) respectively at 4°C overnight. After three washes in TBST, the membranes were blocked using Tris-buffered saline with 0.1% Tween 20 (TBST) and then electrotransferred (Trans-Blot SD semi-dry transfer cell, BioRad, Hercules, CA) to 0.45 μm nitrocellulose membranes (Millipore). The membranes were probed with primary antibody (1/1000 dilution (EPITOMICS, China)) for 1 h at RT and washed 3 times with TBST again. The final detection was visualized by using enhanced chemiluminescence (ECL) (Thermo Scientific, Rockford, IL) Western blotting reagents and images were captured by using ChemiDoc XRS (BioRad). Loading differences were normalized by using an anti-actin antibody with 1/1000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical analysis
All data were from at least 6 rats in each group. The comparisons among multiple groups of the effect of NAC on the protection of BTB after exposures to 40 Gy of SR X-ray were performed by χ² statistic, and P < 0.05 was considered statistically significant. For quantification of Western blot, the proteins bands were densitometrically scanned and compared by using one-way ANOVA, followed by Tukey’s post-hoc test. P < 0.05 was considered statistically significant.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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