

Original Article

Roles of oxidative stress in synchrotron radiation X-ray-induced testicular damage of rodents

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Abstract: Synchrotron radiation (SR) X-ray has characteristic properties such as coherence and high photon flux, which has excellent potential for its applications in medical imaging and cancer treatment. However, there is little information regarding the mechanisms underlying the damaging effects of SR X-ray on biological tissues. Oxidative stress plays an important role in the tissue damage induced by conventional X-ray, while the role of oxidative stress in the tissue injury induced by SR X-ray remains unknown. In this study we used the male gonads of rats as a model to study the roles of oxidative stress in SR X-ray-induced tissue damage. Exposures of the testes to SR X-ray at various radiation doses did not significantly increase the lipid peroxidation of the tissues, assessed at one day after the irradiation. No significant decreases in the levels of GSH or total antioxidation capacity were found in the SR X-ray-irradiated testes. However, the SR X-ray at 40 Gy induced a marked increase in phosphorylated H2AX – a marker of double-strand DNA damage, which was significantly decreased by the antioxidant N-acetyl cysteine (NAC). NAC also attenuated the SR X-ray-induced decreases in the cell layer number of seminiferous tubules. Collectively, our observations have provided the first characterization of SR X-ray-induced oxidative damage of biological tissues: SR X-ray at high doses can induce DNA damage and certain tissue damage during the acute phase of the irradiation, at least partially by generating oxidative stress. However, SR X-ray of various radiation doses did not increase lipid peroxidation.

Keywords: Synchrotron radiation, X-ray, oxidative stress, testes, DNA damage, lipid peroxidation

Introduction

Synchrotron radiation (SR) X-ray is coherent, monochromatic, collimated, and intensely bright light. These properties have enabled the light to have rapidly increasing applications for basic biomedical research as well as great potential for medical applications [1, 2]. For examples, a number of studies have suggested that SR X-ray-based microbeam radiation therapy (MRT) may become a novel approach for treating such cancers as gliomas [3-5]; and SR X-ray-based medical imaging could produce images with exceptional high resolutions [1, 6]. However, in order to apply SR X-ray in medical settings, it is necessary to answer the following important, interrelated questions [7]: 1) What are the mechanisms underlying SR X-ray-induced tissue damage? 2) Are the safe doses of SR X-ray the same

as that of conventional X-ray in medical settings? 3) Do SR X-ray and conventional X-ray impair biological tissues by the same or different mechanisms? So far there have been only few studies on these scientific questions, most of which have studied the safety of MRT on the brain [8]. Our latest study has suggested that NAD⁺ administration can significantly decrease SR X-ray-induced tissue damage [9].

Many studies have indicated that X-ray, as one of the major forms of ionizing radiation, impairs biological tissues through either direct interactions or indirect interactions [10]: In direct interactions, ionizing irradiation attacks macromolecules directly [11]; while in indirect interactions, X-ray impairs tissues indirectly by inducing generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [12]. X-ray can

generate ROS, including hydroxyl radicals, by directly inducing radiolysis of water [13].

There has been no study regarding the roles of ROS in the damaging effects of SR X-ray on biological tissues. However, theoretically, SR X-ray might produce lower levels of lipid peroxidation compared to normal X-ray for the following reasons: It has been indicated that conventional X-ray of high dose rates produces lower levels of chain reactions of lipid peroxidation compared to the X-ray of low dose rates, because the X-ray of high dose rates can produce higher concentrations of lipid peroxides, which would lead to recombination of lipid peroxides thus blocking the propagation of lipid peroxidation [14, 15].

In this study, by using male gonads as a model, we conducted the first study on SR X-ray-induced oxidative damage of biological tissues in the acute phase of the irradiation. Our study has suggested that oxidative stress may play a significant role in certain SR X-ray-induced damage such as double-strand DNA (dsDNA) damage. However, the SR X-ray irradiation at a wide range of radiation doses did not significantly increase lipid peroxidation of the tissues.

Materials and methods

Materials

The chemicals and antibodies used in this study were purchased from Sigma Chemicals (St. Louis, MO, USA) except where noted.

Procedures of animal operation and SR X-ray irradiation

As described previously [9], the experiments were carried out on male SD rats with body weight of 190–220 g at the medical research beamline BL13W in Shanghai Synchrotron Radiation Facility (SSRF). All operative procedures were in conformity with the Guidelines for the Care and Use of Laboratory Animals of Shanghai Jiao Tong University. Briefly, each rat was anesthetized by an intraperitoneal injection of 10% chloral hydrate (0.5 ml per 100 g of body weight), then fixed on one plane of a self-made vertical stereotactic frame, with male gonads hanging out of the frame. The gonads were directly irradiated by SR X-ray. The other plane of frame is covered by a lead sheet to protect other parts of body of the rats from irradiation. After the SR X-ray irradiation, rats were housed in the

animal room at 22–24°C with 12-hour light/dark cycle and free access to food and water. As described previously [9], we calculated the radiation doses based on the air kerma of the SR X-ray at the entrance of the tissues.

Western blot assay on phosphorylated histone family 2A variant (H2AX)

As described previously [9], tissue lysates were centrifuged at 12,000 g for 20 min at 4°C. After quantifications of the protein samples using BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA), 30 µg of total protein was electrophoresed through a 10% SDS-polyacrylamide gel, and then wet electrotransferred to 0.45 µm nitrocellulose membranes (Millipore, CA, USA). The blots were incubated overnight at 4°C with a monoclonal anti-phospho-histone H2AX (Millipore, Billerica, MA, USA) (1:200 dilution), then incubated with a rabbit anti-goat polyclonal HRP-conjugated secondary antibody (EPITOMICS, Hangzhou, Zhejiang Province, China). Protein signals were detected using an enhanced chemiluminescence detection system (Pierce Biotechnology, Rockford, IL, USA). An anti-β-actin antibody with 1:1000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to normalize sample loading and transfer. The intensities of the bands were quantified by densitometry using Gel-Pro Analyzer.

Hematoxylin staining

As described previously [9], tissue cryosections were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 10 mins at RT, washed in PBS, followed by staining with hematoxylin dye (Beyotime, Haimen, Jiangshu Province, China) for approximately 10 min at RT. After the slides were washed in water for 30 min, the slides were mounted with an aqueous mounting medium and viewed under a Leica microscope, which is interfaced with a digital camera. To count the cell layers of seminiferous tubules, the slides were examined at 200× magnification. Cell layers of the seminiferous tubules in each cryosection of the testis were counted in a blinded fashion, the average of which is defined as the 'cell layer' of the seminiferous tubules.

TBARS assay

The levels of thiobarbituric acid reactive substances (TBARS) in the testes were determined

by using a commercially available kit (Cayman Chemical, Ann Arbor, MI). In brief, the tissues of the testes were weighed and homogenized. The assay was performed using a plate reader according to the manufacturer's protocol.

Assays of the levels of GSH and total antioxidant capacity (T-AOC)

The GSH and T-AOC level of the testes were measured by using commercially available kits (Jiancheng Bioengineering Institute, Nanjing, P.R. China). The assay was performed using a plate reader according to the manufacturer's protocol.

Statistical analyses

All data are presented as mean $\pm$ SE. Data were assessed by one-way ANOVA, followed by Student-Newman-Keuls post hoc test. *P* values less than 0.05 were considered statistically significant.

Results

Lipid peroxidation is one of the key forms of oxidative damage in biological tissues [16]. TBARS, including malondialdehyde (MDA), are the major products generated in the chain reactions of lipid peroxidation [16]. We applied TBARS assay to determine the effects of SR X-ray on the lipid peroxidation of the testes. The gonads of male rats were exposed to SR X-ray at radiation doses of 0, 0.5 Gy, 1.3 Gy, 4 Gy and 40 Gy. One day after the exposures, the levels of TBARS of the testes were determined. We found that exposures of the testes to SR X-ray at the doses ranging from 0.5 to 40 Gy did not significantly increase or decrease the TBARS levels (**Figure 1**).

GSH is the key antioxidation factor in cells. It has been reported that mild oxidative stress can induce an adaptive response by increasing intracellular GSH levels, while severe oxidative stress can lead to decreased levels of GSH [17, 18]. In this study we determined the effects of SR X-ray on the levels of GSH in the testes. The gonads of male rats were exposed to SR X-ray at radiation doses of 0, 0.5 Gy, 1.3 Gy, 4 Gy and 40 Gy. One day after the exposures, the levels of GSH of the testes were determined. We did not find that the SR X-ray at any radiation doses significantly affected the GSH levels (**Figure 2**).

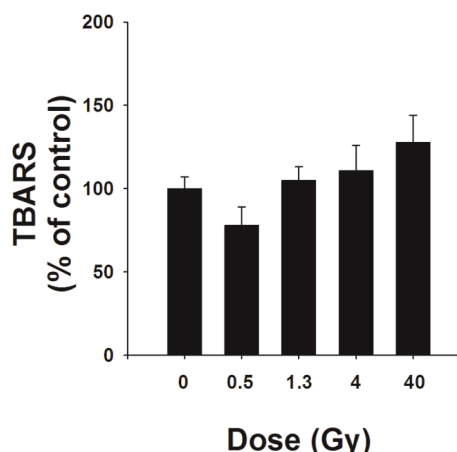


Figure 1. Exposures of the testes of rats to various doses of SR X-ray did not significantly increase the TBARS levels of the testes. The gonads of male rats were exposed to SR X-ray at radiation doses of 0, 0.5 Gy, 1.3 Gy, 4 Gy and 40 Gy. One day after the SR X-ray exposures, the TBARS levels of the testes were determined. N= 21-22 rats for each experimental condition.

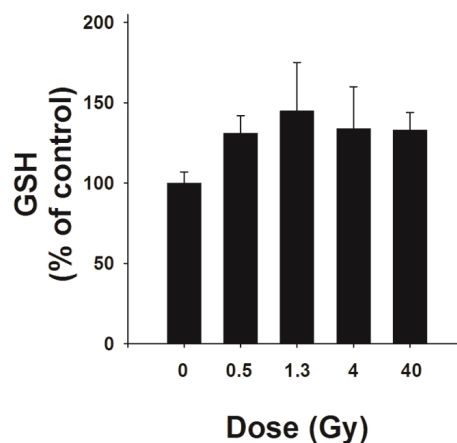


Figure 2. Effects of SR X-ray irradiation on the levels of GSH in the testes. The gonads of male rats were exposed to SR X-ray at doses of 0, 0.5 Gy, 1.3 Gy, 4 Gy and 40 Gy. One day after the SR X-ray exposures, the GSH levels of the testes were determined. N= 8-17 rats for each experimental condition.

We further determined the effects of SR X-ray on the levels of total antioxidant capacity (T-AOC) of the testes. No significant effect of SR X-ray at any radiation doses on the levels of the T-AOC was observed (**Figure 3**).

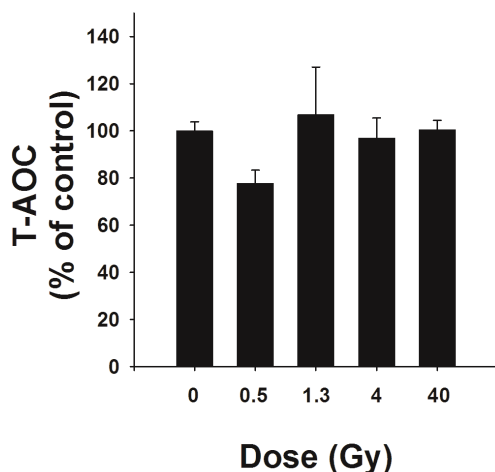


Figure 3. Effects of SR X-ray irradiation on the levels of total antioxidant capacity (T-AOC) in the testes. The gonads of male rats were exposed to SR X-ray at doses of 0, 0.5 Gy, 1.3 Gy, 4 Gy and 40 Gy. One day after the SR X-ray exposures, the T-AOC levels of the testes were determined. N= 8-17 rats for each experimental condition.

It is established that double-strand DNA (dsDNA) damage can activate p53, which can induce increased expression of the pro-apoptotic protein Bax [19]. Western blot assays of phosphorylated H2AX (γ H2AX) – a hallmark of dsDNA, showed that 40 Gy SR X-ray induced an increase in γ H2AX, which was attenuated by i.v. administration of 25 mg/kg NAC (Figure 4A). Quantifications of the results showed that the SR X-ray induced a significant increase in γ H2AX, which was significantly attenuated by the administration of 25 mg/kg NAC (Figure 4B).

We also applied hemotoxylin staining to determine the effects of SR X-ray on the tissue damage of testes. We found that 40 Gy SR X-ray induced a significant decrease in the cell layers of the seminiferous tubules, which was significantly attenuated by the administration of 25 mg/kg NAC (Figure 5).

Discussion

Our study has provided the first characterization of the roles of oxidative stress in SR X-ray-induced tissue damage. The key experimental results of our study include: 1) Oxidative stress play a significant role in the dsDNA damage

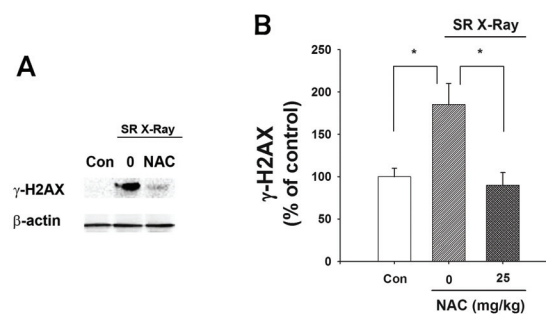


Figure 4. (A) Effects of 25 mg/kg NAC on SR X-ray-induced dsDNA damage of the testes, as assessed by Western blot. (B) Quantifications of the results of Western blot showed that 40 Gy SR X-ray significantly increased double-strand DNA damage, which was significantly attenuated by administration of the NAC administration. The testes of rats were exposed to 1.3, 4 or 40 Gy SR X-ray. For the rats exposed to 40 Gy of SR X-ray, a subset of the rats was administered intravenously with PBS or NAC. One day after the SR X-ray exposures, the effects of NAC administration on SR X-ray-induced dsDNA damage was assessed by Western blot using anti- γ H2AX antibody. N= 12 rats for each experimental condition. * $p < 0.05$.

induced by a high dose of SR X-ray irradiation; 2) oxidative stress play a significant role in such SR X-ray-induced tissue damage as the decrease in the cell layer number of the seminiferous tubules of the testes; and 3) the SR X-ray at various radiation dose can induce neither a significant increase in lipid peroxidation nor significant decreases in the levels of GSH and total antioxidant capacity of the tissues.

Due to the characteristic properties of SR X-ray, such as high intensity and coherence, SR X-ray has great potential for applications in medical imaging and medical treatment of cancer [3-5]. However, in order to apply SR X-ray in medical settings, it is required to determine if the mechanisms underlying SR X-ray-produced tissue damage are the same or different from that of conventional X-ray, based on which the safety standard of SR X-ray in medical settings may be established. However, so far there has been little information regarding the mechanisms underlying SR X-ray-produced tissue injury.

Many studies have indicated that oxidative damage is one of the key mechanisms underlying the indirect damaging effects of normal X-ray on

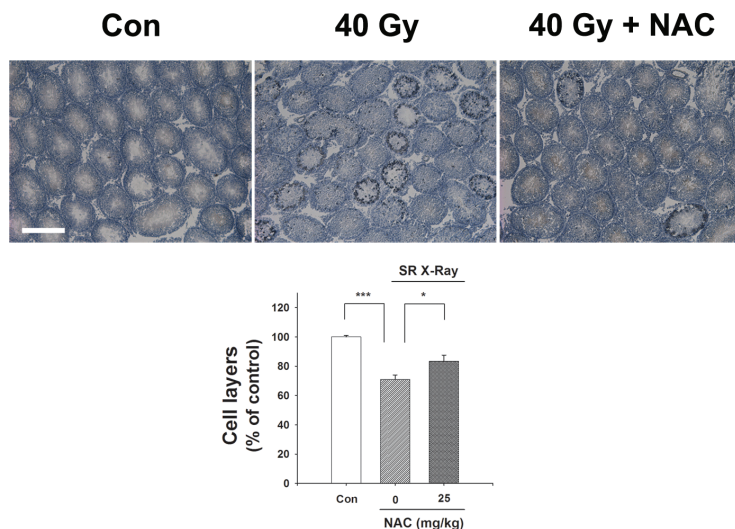


Figure 5. Effects of NAC administration on the SR X-ray-induced changes of the cell layers of the seminiferous tubules of rat testes, assessed by hematoxylin staining (A). Quantifications of the cell layers indicate that NAC administration significantly attenuated SR X-ray-induced decreases in the cell layers of the seminiferous tubules (B). The testes of rats were exposed to 1.3, 4 or 40 Gy SR X-ray. For the rats exposed to 40 Gy of SR X-ray, a subset of the rats was administered intravenously with PBS or NAC. One day after the SR X-ray exposures, hematoxylin staining was applied to determine the effects of NAC on the SR X-ray-induced changes of the cell layers of testes. N= 10 - 13 rats for each experimental condition. * $p < 0.05$.

biological tissues [20-22]: Normal X-ray irradiation can generate ROS by directly inducing radiolysis of water [13], which can impair various cellular components including DNA and membrane phospholipids. ROS can produce tissue damage by multiple mechanisms, including disrupting calcium homeostasis, inducing cell apoptosis and necrosis pathways, activating poly(ADP-ribose) polymerase-1, and impairing mitochondria [23, 24]. However, there is evidence suggesting that, compared to its role in the conventional X-ray-induced tissue damage, ROS might play different roles in SR X-ray-induced tissue damage: Lipid peroxidation is one of the key forms of oxidative damage in biological tissues [16]. It has been indicated that the conventional X-ray of high dose rates can produce higher concentrations of lipid peroxides, which would lead to recombination of lipid peroxides thus blocking the propagation of lipid peroxidation [14, 15]. Therefore, the conventional X-ray of high dose rates may produce lower levels of chain reactions of lipid peroxidation compared to the X-ray of low dose rates. Therefore, we hypothesized that due to the high dose rate of SR X-ray, lipid peroxidation may play a relatively minor role in SR X-ray-produced tissue damage.

Our study has shown that the SR X-ray irradiation of various doses ranging from 0.5 to 40 Gy did not significantly affect the levels of lipid peroxidation of the testes, thus supporting our hypothesis proposing that lipid peroxidation may play a relatively minor role in SR X-ray-induced

tissue damage. In other words, our study has provided the first evidence suggesting that there may be differences in the mechanisms underlying SR X-ray-induced tissue damage compared to the mechanisms underlying conventional X-ray-induced tissue damage.

DNA damage belongs to the pivotal damage produced by X-ray irradiation [11]. Both single-strand DNA damage and dsDNA damage can initiate cell death pathways [25-27]. It has been reported that dsDNA damage can induce apoptosis by activating such pathways as p53-dependent pathway [19, 27, 28]. Our observation that NAC can prevent SR X-ray-induced increases in dsDNA damage suggests that oxidative stress plays a significant role in certain SR X-ray-induced damage. The protective effects of the NAC administration on dsDNA damage may lead to prevention of the dsDNA-initiated cell death pathway, which may partially underlie our observations that the NAC administration can attenuate the SR X-ray-induced reduction in the cell layers.

GSH is the key antioxidation factor in cells. Our study has found that the SR X-ray of a wide range of doses did not significantly affect the GSH levels or the levels of total antioxidation capacity of the testes. This observation does not contradict to our observations suggesting that ROS plays significant roles in such SR X-ray-induced tissue damage as dsDNA damage, due to the following reasons: Significant decreases in the levels of GSH can be generated only by severe

oxidative stress [17, 18]. Combining the observation that SR X-ray did not significantly affect the GSH levels of the tissues and the observation that oxidative stress plays significant roles in SR X-ray-induced dsDNA damage, our study has suggested that SR X-ray at high doses could produce mild or moderate levels of oxidative stress, which is not severe enough to decrease the GSH levels or increase the lipid peroxidation levels of the tissues.

In the current study we focused on the acute effects of SR X-ray exposures on the oxidative stress of the testes, by conducting out study mainly at one day after the SR X-ray exposures. The rationale for this experimental protocol is: Because X-ray can induce rapid generation of ROS by inducing radiolysis of water [13], it is necessary to study the acute effects of SR X-ray exposures on the oxidative stress in order to determine the roles of ROS in the SR X-ray-induced tissue injury. In contrast, studies on the roles of oxidative stress at late time points after irradiation could be less significant for determining if ROS initiates the tissue injury after X-ray irradiation, because the oxidative stress at the later time points may be only a secondary event in the cascade initiated by the primary mechanisms of SR X-rays-induced tissue damage.

In this study we used male gonads of rats as the model to study the mechanisms underlying the SR X-ray-induced injury of biological tissues. Because gonads are one of the most radiosensitive organs, the study on the mechanisms of irradiation injury of gonads would provide valuable information for establishing the safety standard of SR X-ray in medical settings. To our knowledge, our current study is the one of the first that studies the damaging effects of SR X-ray on gonads. However, since it remains possible that the mechanisms underlying the SR X-ray-induced injury of different organs may be differential, it is warranted to use other organs to study the mechanisms underlying the SR X-ray-induced injury of biological tissues in the future.

In summary, our study has shown that SR X-ray-induced dsDNA damage and decreases in the cell layers of semiferous tubules can be significantly attenuated by NAC administration. These results have suggested that oxidative stress plays significant roles in certain SR X-ray-induced tissue damage. The observation has

also suggested that NAC may be used to decrease SR X-ray-produced damage of normal tissues, which would further enhance the potential of using SR X-ray in medical settings. In addition, our study regarding the effects of SR X-ray on dsDNA damage has provided the first evidence suggesting that there may be differences in the mechanisms underlying SR X-ray-induced tissue damage compared to the mechanisms underlying conventional X-ray-induced tissue damage. So far there has been little information regarding the mechanisms underlying the effects of SR X-ray on normal tissues. Our findings regarding the roles of oxidative stress in SR X-ray-induced tissue damage have suggested the first mechanism of the tissue damage, which have also provided a valuable basis for future investigation on these mechanisms.

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