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Original Article

Dose-rate plays a significant role in synchrotron radiation X-ray-induced damage of rodent testes

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Abstract: Synchrotron radiation (SR) X-ray has significant potential for applications in medical imaging and cancer treatment. However, the mechanisms underlying SR X-ray-induced tissue damage remain unclear. Previous studies on regular X-ray-induced tissue damage have suggested that dose-rate could affect radiation damage. Because SR X-ray has exceedingly high dose-rate compared to regular X-ray, it remains to be determined if dose-rate may affect SR X-ray-induced tissue damage. We used rodent testes as a model to investigate the role of dose-rate in SR X-ray-induced tissue damage. One day after SR X-ray irradiation, we determined the effects of the irradiation of the same dosage at two different dose-rates, 0.11 Gy/s and 1.1 Gy/s, on TUNEL signals, caspase-3 activation and DNA double-strand breaks (DSBs) of the testes. Compared to those produced by the irradiation at 0.11 Gy/s, irradiation at 1.1 Gy/s produced higher levels of DSBs, TUNEL signals, and caspase-3 activation in the testes. Our study has provided the first evidence suggesting that dose-rate could be a significant factor in SR X-ray-induced tissue damage, which may establish a valuable base for utilizing this factor to manipulate the tissue damage in SR X-ray-based medical applications.

Keywords: Synchrotron radiation X-ray, dose-rate, tissue damage, rodent testes

Introduction

Dose-rate is one of the fundamental properties of radiation. Multiple *in vitro* and *in vivo* studies on regular X-ray-produced radiation damage have suggested that dose-rate could significantly affect radiation damage on both normal and tumor tissues [1]. Previous cell culture studies have suggested that dose-rate produces contrasting effects on DNA damage and lipid peroxidation [2-7]. At the same dosage, increasing dose-rate leads to increased DNA damage, while it produces decreased lipid peroxidation.

Increasing evidence has suggested that SR X-ray may have important applications in clinical medicine [8]: SR X-ray-based medical imaging may produce medical images with significantly enhanced resolutions [8], and SR X-ray-based microbeam radiation therapy (MRT) may be used as a powerful approach for treating gliomas [9-11]. However, there have been only several studies regarding the mechanism underlying SR X-ray-produced tissue damage. Because the dose-rate of SR X-ray is dramatically higher compared with that of regular X-ray, it is of great interest to determine if the exceedingly high dose-rate of SR X-ray may become an important factor in the SR X-ray-produced radiation injury.

In this study, we used rodent testes, a widely used model for radiobiology research, to determine the role of dose-rate in SR X-ray-induced tissue damage. Our study has provided the first evidence suggesting that increasing dose-rate can exacerbate SR X-ray-induced DNA damage and apoptotic changes during acute phase of irradiation. These results have suggested that dose-rate may be modulated to manipulate SR X-ray-produced tissue damage in its medical applications.

Materials and methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Shanghai Administrative Committee. All of the animal protocols were approved by the Animal Study Committee of the School of Biomedical Engineering, Shanghai Jiao Tong University (Permit Number: 2012001). All surgical procedures were performed by Ban Wang (Qualification Certificate Number: 14080366) under chloral hydrate anesthesia, in which all efforts were made to minimize animal suffering. Rats were sacrificed by cervical dislocation at a designed time as shown in Results.

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) except where noted. Male Sprague-Dawley rats (SLAC, Shanghai, China) weighing from 190 g to 220 g were used in this study. The animals were housed under the same conditions as described previously [12]. All animal procedures were conducted following the protocol approved by the institutional Animal Care and Use Committee, School of Biomedical Engineering, Shanghai Jiao Tong University.

Exposures of male gonads of rats to SR X-Ray irradiation

All animal surgeries were performed under chloral hydrate-induced anesthesia. Rodent testes were irradiated by SR X-ray at beam line station BL13W1 of Shanghai Synchrotron Radiation Facility (SSRF) at the dose-rate of either 0.11 Gy/s or 1.1 Gy/s. The radiation dosage was either 1 Gy or 4 Gy, using 33.7 keV SR X-ray. To change the dose-rate of SR x-ray, we used carbon filter and adjusted the angel of piezo control. The gonads of the anesthetized rats were exposed to SR X-ray, while other parts of the body were protected by lead sheets [12].

Sample collections

The rats were sacrificed at 1 day after SR X-ray irradiation. The testes were harvested and snap frozen in liquid nitrogen. Then the testes were stored at -80°C until further analysis.

Calculations of radiation dose

The radiation dose was calculated as previously described [12]. We used an ionization chamber to determine the photon flux of SR X-ray, and then the photon flux was used to calculate the air kerma at the entrance of the tissues, which can be converted to the average radiation dose of the samples. The average radiation dose is:

$$D_{average} \approx X_{ak} \frac{(\mu_{en}/\rho)_{tissue}}{(\mu_{en}/\rho)_{air}} [\frac{1}{T} \int_0^T e^{-utz} dz]$$

Where X_{ak} = Air kerma, (μ_{en}/ρ) tissue = Mass Energy-absorption coefficient of tissue (testis) for photons of 33.7 keV, (μ_{en}/ρ) air = Mass Energy-absorption coefficient of air for photons of 33.7 keV, T = Sample thickness, μ_t = Tissue linear attenuation coefficient for photons of 33.7 keV.

TUNEL assay

TUNEL assays (ApopTag® Kit, CHEMICON) were conducted to assess apoptosis-like DNA fragmentation in testes cyrosections according to the manual provided with the kit. The seminiferous tubule containing three or more TUNEL positive nuclei was considered a TUNEL-positive tubule. At least 150 tubules were assessed for each rat. 10~14 rats were used for each experimental condition.

Caspase-3 activity assay

Caspase-3 activity assays (Beyotime, Haimen, P.R China) were conducted according to the manufacture's protocol. The activity is determined by detecting the production of the p-nitroanilide, which is cleaved from acetyl-Asp-Glu-Val-Asp p-nitroanilide by cleaved caspase-3. Samples were measured with an ELISA reader at the absorbance of 405 nm. Results were normalized to protein concentrations as determined by Bradford Protein Assays (Beyotime, Haimen, P.R China). 5-6 rats were used for each experimental condition.

Immunofluorescence

Ten μm cryosections were fixed in 4% PFA for 10 min at room temperature (RT). After washed one time in PBS, the sections were incubated in 2 μg/ml monoclonal anti-phospho-histone H2AX antibody (Millipore, Billerica, MA, USA) at

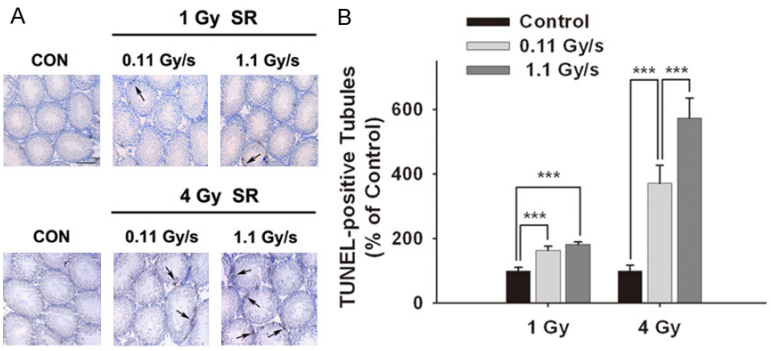

Figure 1. TUNEL signals induced by SR X-ray irradiation at different dose-rates one day after irradiation. A. TUNEL assays were performed to determine apoptosis-like changes in the testes induced by 1 Gy or 4 Gy SR-X ray irradiation. The arrows indicate the TUNEL positive signals. B. Quantifications of the TUNEL signals showed that 4 Gy SR X-ray irradiation at the dose-rate of 1.1 Gy/s induced higher levels of apoptosis-like changes compared to those induced by the 4 Gy SR X-ray irradiation at 0.11 Gy/s. Error bars indicate the standard error of the mean (SEM), N = 10-14 rats for each experimental condition. ***P < 0.001, BAR = 200 μm.

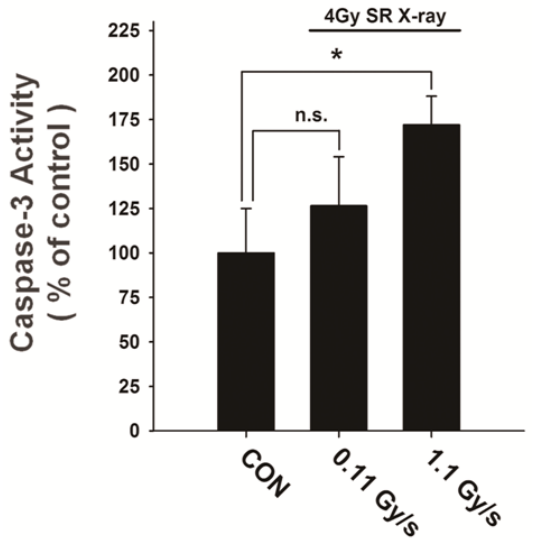

Figure 2. Caspase-3 activation induced by SR X-ray irradiation at different dose-rates 12 hrs after irradiation. Caspase-3 activity assays showed that 4 Gy SR X-ray at 1.1 Gy/s significantly increased caspase-3 activities in the testes, while 4 Gy SR X-ray at 0.11 Gy/s did not. Error bars indicate the SEM, N = 5-6 rats for each experimental condition. n.s., P > 0.05; *P < 0.05.

4°C over night. Then the sections were incubated in Alexa Fluor 568 goat anti-mouse IgG (1:500 dilution, Molecular Probes, Eugene, Oregon, USA) at RT for 1 hr and stained in 0.2% 4',6-diamidino-2-phenylindole (DAPI). After mouted, the sections were imaged using a Leica confocal microscopy. To determine the potential differences in the immunofluorescence signals among various groups, the immunofluorescence signals were judged by at least two different people in double-blinded fashion. 6~9 rats were used for each experimental condition.

Western blot

Eighty μg of protein was electrophoresed, wet electrotransferred to 0.45 μm nitrocellulose membranes (Millipore, CA, USA) and incubated with a monoclonal anti-phospho-histone H2AX (1:500 dilution, Millipore, Billerica, MA, USA) or an anti-tubulin antibody (1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After incubated with HRP-conjugated secondary antibody (EPITOMICS, Hangzhou, Zhejiang Province, China), bands were detected by An ECL detection system (Pierce Biotechnology, Rockford, IL, USA) and quantified by densitometry using Gel-Pro Analyzer. 7~9 rats were used for each experimental condition.

Statistical analyses

All data are presented as mean ± 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

SR X-ray irradiation at higher dose-rate induced greater TUNEL signals in rodent testes

One day after irradiation, TUNEL assays were performed to determine apoptosis-like changes induced by 1 or 4 Gy SR-X ray (**Figure 1A**). At the dosage of 4 Gy - a widely used dosage in irradiation studies using testes [13], the SR X-ray irradiation at the dose-rate of 1.1 Gy/s induced significantly higher levels of TUNEL signals compared with those induced by SR X-ray irradiation at the dose-rate of 0.11 Gy/s (**Figure 1B**).

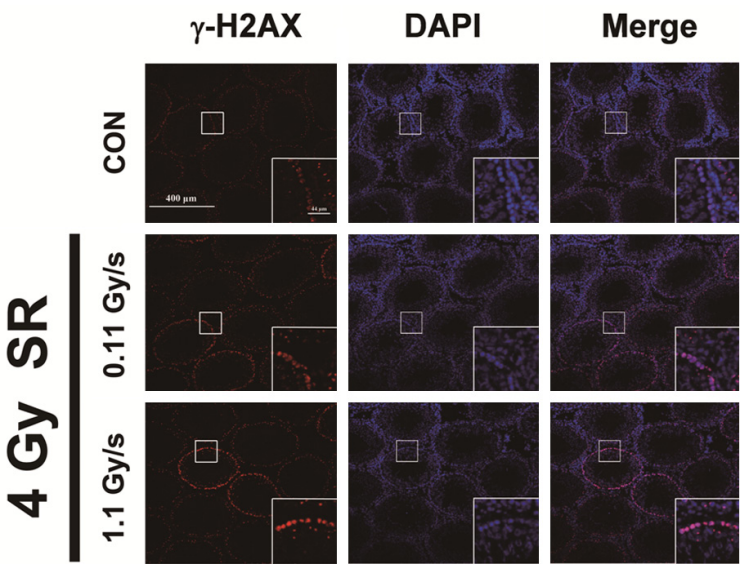

Figure 3. γ-H2AX induced by SR X-ray irradiation at different dose-rates one day after irradiation. Immunostaining assays showed that γ-H2AX levels in the testes irradiated by 4 Gy SR X-ray at 1.1 Gy/s were significantly higher than those in the testes irradiated by 4 Gy SR X-ray at 0.11 Gy/s. DNA was counterstained with DAPI. N = 6-9 rats for each experimental condition.

SR X-ray irradiation at higher dose-rate induced greater caspase-3 activation in rodent testes

12 hrs after the irradiation, caspase-3 activity assays showed that 4 Gy SR X-ray at 1.1 Gy/s significantly increased caspase-3 activities in the testes. However, 4 Gy SR X-ray at 0.11 Gy/s did not induce significant increases in caspase-3 activities compared with control (**Figure 2**).

SR X-ray irradiation at higher dose-rate induced higher γ-H2AX levels in rodent testes

One day after irradiation, we determined the effects of the irradiation of 4 Gy SR X-ray at the dose-rate of either 0.11 Gy/s or 1.1 Gy/s on DSBs, as assessed by immunostaining of γ-H2AX [2, 14]. The immunostaining assays showed that 4 Gy SR X-ray irradiation at 1.1 Gy/s induced stronger γ-H2AX signals compared with those induced by the SR X-ray irradiation at 0.11 Gy/s (**Figure 3**). Western blot were also conducted to determine the radiation-induced γ-H2AX levels in the testes, showing that 4 Gy SR X-ray irradiation at 1.1 Gy/s induced higher γ-H2AX levels compared with those induced by the SR X-ray irradiation at 0.11 Gy/s (**Figure 4**).

Discussion

The major findings of this study include: First, at the same dosage, the SR X-ray at higher dose-rate produces higher levels of DNA damage during acute phase of irradiation; and second, the SR X-ray at higher dose-rate produces higher levels of apoptotic changes during acute phase of irradiation. Collectively, our study has provided the first evidence suggesting that dose-rate could be an important factor determining SR X-ray-induced tissue injury.

In the previous studies regarding dose-rate effect of ionized radiation-induced injuries, the low dose-rate irradiation was delivered in minutes or days [1, 2]. Considering the intensity of SR X-ray is higher than conventional X-ray, we explored the dose-rate effect at the higher dose-rate than before, which is 0.11 Gy/s to 1.1 Gy/s, finding that 4 Gy SR X-ray delivered in different dose-rate induced significant differences in testicle tissue injuries.

Several studies have indicated that increasing dose-rate leads to increased DNA damage in Human fibroblast cells and Chinese Hamster V79 Cells [2, 3]. The proposed mechanism is that the ionizing radiation at high dose-rate can produce extensive DNA damage at certain durations of time which surpasses the DNA repair capacity of the cells [2]. In contrast, several studies have shown that increasing dose-rate at identical doses leads to a decrease in radiation-induced lipid peroxidation [4-7]. Theoretical explanations for this inverse dose-rate effect are [4, 7]. The propagation of the chain reactions of lipid peroxidation is mediated by peroxyl radicals, which is terminated due to elimination of radical species by radical-radical interactions. Thus, the high dose-rate would lead to increased levels of radicals at certain durations, resulting in increased radical-radical interactions and accelerated termination of lipid peroxidation.

Our current study has suggested that the overall effect of increasing dose-rate of SR X-ray is

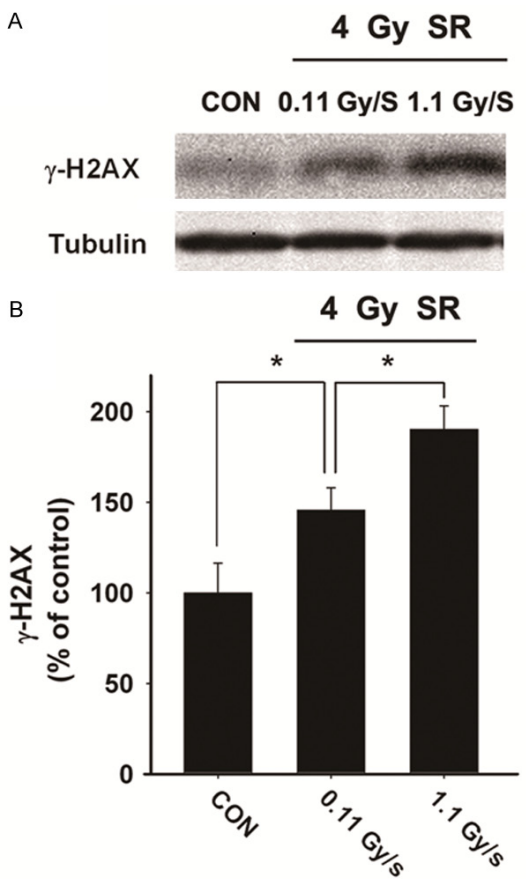

Figure 4. γ-H2AX induced by SR X-ray irradiation at different dose-rates one day after irradiation. A. Ds-DNA damage of the testes irradiated by 4Gy SR X-ray at different dose-rates, as assessed by Western blot. B. Quantifications of the results of Western blot showed that the 4 Gy SR X-ray irradiation at 1.1 Gy/s induced higher γ-H2AX levels in the testes than 4 Gy SR X-ray irradiation at 0.11 Gy/s did. Error bars indicate SEM, N = 7-9 rats for each experimental condition. *$P < 0.05$.

that high dose-rate leads to increased tissue damage during the acute phase of SR X-ray irradiation, as indicated by the increases in both TUNEL staining signals and caspase-3 activity. Considering our previous studies suggesting a key role of oxidative stress in SR X-ray-induced damage of testes [14, 15], future studies are warranted to investigate the roles of oxidative stress in the tissue damage induced by SR X-ray of various dose-rates.

It is established that DSBs is a critical factor mediating ionizing radiation-induced cell death [16]. DSBs can induce increased p53-dependent expression of such apoptotic factors as Bax, thus leading to such apoptotic changes as caspase-3 activation [17]. Similar with the potential mechanisms underlying the findings regarding the roles of dose-rate in regular X-ray-induced DNA damage [2], higher dose-rate of the SR X-ray irradiation may lead to higher rate of DNA damage which may surpass the DNA repair capacity of cells.

Multiple studies have suggested that SR X-ray-based MRT may be a promising approach for treating gliomas [10, 11]. The possible mechanisms underlying the therapeutic potential of MRT include: First, due to the high Peak-to-Valley Dose Ratio, MRT can deliver a high uniform dose deposition at tumors whereas surrounding normal tissues are irradiated by well tolerated dose [18]; and second, microvasculature in normal tissue seems to effectively repair itself after irradiation, but the microvasculature in tumors fails to do so [19]. Based on our current study, we propose a new potential mechanism accounting for the efficacy of SR X-ray in treating glioma: Compared to regular X-ray, the high dose-rate of SR X-ray might lead to increased tumor cell death at the same dosage. Although our dose-rate is significantly lower than the dose-rate used in MRT [18, 20], our study is the first one that determines the dose-rate effects of SR X-ray irradiation on tissue damage. It is our belief that our study would be valuable for the future studies regarding the dose-rate effects of SR microbeam X-ray on tissue damage. Future studies are warranted to test the validity of our proposal.

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Disclosure of conflict of interest

None.

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