Original Article

Simulated Microgravity Conditions and Carbon Ion Irradiation Induce Spermatogenic Cell Apoptosis and Sperm DNA Damage*

LI Hong Yan[1,2,3,4], ZHANG Hong[1,2,3,#], MIAO Guo Ying[1,2,3,4], XIE Yi[1,2,3], SUN Chao[1,2,3,4], DI Cui Xia[1,2,3], LIU Yang[1,2,3], LIU Yuan Yuan[1,2,3], ZHANG Xin[1,2,3], MA Xiao Fei[1,2,3], XU Shuai[1,2,3,4], GAN Lu[1,2,3], and ZHOU Xin[1,2,3]

1. Department of Heavy Ion Radiation Medicine, Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou 730000, Gansu, China; 2. Key Laboratory of Heavy Ion Radiation Biology and Medicine of Chinese Academy of Sciences, Lanzhou 730000, Gansu, China; 3. Key Laboratory of Heavy Ion Radiation Medicine of Gansu Province, Lanzhou 730000, China; 4. University of Chinese Academy of Sciences, Beijing 100049, China

Abstract

Objective To investigate the effect of simulated microgravity and carbon ion irradiation (CIR) on spermatogenic cell apoptosis and sperm DNA damage to the testis of male Swiss Webster mice, and assess the risk associated with space environment.

Methods Sperm DNA damage indicated by DNA fragmentation index (DFI) and high DNA stainability (HDS) was measured by sperm chromatin structure assay (SCSA). Apoptosis of spermatogenic cells was detected by annexin V-propidium iodide assay. Bax (the expression levels of p53) and proliferating cell nuclear antigen (PCNA) were measured by immunoblotting; p53 and PCNA were located by immunohistology.

Results HDS, DFI, apoptosis index, and the expression levels of p53 and Bax were detected to be significantly higher in the experimental groups (P<0.05) compared with those in the control group; however, the PCNA expression varied to a certain degree. p53- and PCNA- positive expression were detected in each group, mainly in relation to the spermatogonic cells and spermatocytes.

Conclusion The findings of the present study demonstrated that simulated microgravity and CIR can induce spermatogenic cell apoptosis and sperm DNA damage. Sperm DNA damage may be one of the underlying mechanisms behind male fertility decline under space environment. These findings may provide a scientific basis for protecting astronauts and space traveler's health and safety.

Key words: Simulated microgravity; Carbon ion irradiation; Spermatogenic cells apoptosis; Sperm DNA damage

Biomed Environ Sci, 2013; 26(9):726-734 *doi: 10.3967/0895-3988.2013.09.003* *ISSN:0895-3988*

www.besjournal.com(full text) *CN: 11-2816/Q*

*This research was supported by the Knowledge Innovation Project of the Chinese Academy of Sciences (KJCX2-YW-L08), the National Basic Research Program of China (2010CB834202), the National Natural Science Foundation of China (10835011), the Scientific Technology Research Projects of Gansu Province (0702NKDA045, 0806RJYA020).

#Correspondence should be addressed to ZHANG Hong. Tel: 86-931-4969344, Fax: 86-931-8272100. E-mail: zhangh@impcas.ac.cn

Biographical note of the first author：LI Hong Yan, male, born in 1985, Ph.D. candidate, majoring in radiation medicine/biology.

Received: September 2, 2012; Accepted: March 30, 2013

INTRODUCTION

The integrity of chromosomal DNA in a sperm is a prerequisite for normal fertilization and transmission of paternal genetic information[1]. Although fertilization may also occur when sperm DNA is damaged, an abnormal sperm chromatin structure is reported to have been negatively correlated with the fertility and subsequent embryo development[2]. The sperm chromatin structure assay (SCSA), which has been used for more than two decades[3], has been proven to be a sensitive and precise tool to detect sperm DNA integrity and damage by flow cytometry[4] in the toxic environment, such as radiation, heat, and industrial chemicals which damage different types of testicular germ cells[5]. SCSA which uses parameters including sperm DNA fragmentation index (DFI) and high DNA stainability (HDS) sperm index[5] is considered the gold standard to detect the sperm DNA damage with the characteristic of high-throughput and stability[2,6]. DFI is the percentage of damaged sperm DNA divided by the total sperm DNA, which reflects the loss of sperm DNA. HDS refers to the high percentage of stained sperm divided by the total sperm, in which the not fully condensed core represents the immature sperm[7]. The sperm DNA damage may be induced by spermatogenic cells apoptosis during spermatogenesis, or may be directly induced by environmental toxins.

The space has a strong radiation and belongs to the microgravity environment. The main factors that affect biological cells in space environment are microgravity and space radiation[8]. Numbers of studies have reported that negative effects of gravity on biological reproduction and development exist in terms of damaging male reproductive organs. Microgravity can cause testicular atrophy to reduce the level of testosterone secretion, and promote testicular cells apoptosis[9], thereby affecting male fertilization[10]. In addition, oxidative damage in testicular tissues could be induced by CIR, resulting in cell cycle arrest, change in hormone levels, DNA strand breaks and apoptosis rate increase[11].

In the present study, the tail suspension model in the ground state was used to simulate microgravity environment[12-13] and carbon ion beam irradiation was used to investigate the effect of microgravity and space irradiation on the male testis spermatogenic cell apoptosis, proliferation, and sperm DNA damage. This study has laid down a sound basis for further investigating spermatogenic cell apoptosis and sperm DNA damage induced by simulated microgravity and heavy ion irradiation, and has also provided relevant information for protecting the health and safety of astronauts and space travelers.

MATERIALS AND METHODS

Establishment of Simulated Microgravity Model of Mice

A total of 42 adult male Swiss Webster mice (Lanzhou University School of Medicine, China) weighing 30-35 g (about 10-week-old) were used. All feeding procedures were approved by Lanzhou University School of Medicine. The animals were housed in individual cages in a temperature controlled room at 22±2 °C, 60%±10% humidity under 12:12 h light-dark cycle, and provided with standard food pellet and water available adlibitum[14]. The animals were randomly assigned into two groups: no suspended mice group as control (12 mice) and tail suspended mice as microgravity group (30 mice). The tail suspension (SUS) technique was used to simulate microgravity. Briefly, a strip of elastic tape was applied to the surface of the tail. A clip at the end of the tape was fastened to an overhead bar and adjusted to maintain the mice at a 30° head-down tilt, with the hind limbs being elevated above the floor of the cage and the front paws touching the floor. The mice were subjected to SUS for 7 days[12-13].

Irradiation Procedure

The mice in the microgravity group (30 mice) were randomly divided into five groups: SUS group, (SUS+0.25 Gy) group, (SUS+0.5 Gy) group, (SUS+1 Gy) group and (SUS+2 Gy) group; the mice in the unsuspended group was randomly divided into the control group and 2 Gy group (IR), with equal number of mice in each group. Mice were controlled in a chamber and whole-body irradiated with carbon ion beam at 200 MeV/U while 31.3 keV/μm beam entrance at a dose rate approximately 0.5 Gy/min in the Heavy Ion Research Facility in Lanzhou (HIRFL, Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou, China). The carbon ion is equipped with a passive beam delivery system[15]. Data were controlled automatically by a microcomputer during irradiation. Particle influence was determined by an air-ionization chamber signal

according to the calibration of the detector (PTW-UNIDOS, PTW-Freiburg Co., Wiesbaden, Germany).

Quantification of Testes by Histological Evaluation

Six mice from each group were used after 24 h irradiation. Mice were killed using cervical dislocation. The epididymides and testes of each mouse were taken out. The fat and connective tissues adhering to testis were removed. The left testes were frozen at -80 °C, and the right testes were fixed in 4% paraformaldehyde (4 g/100 mL) with 0.01 mol/L phosphate buffer (pH 7.4) at room temperature for 24 h preparing for paraffin section. The fixed testes were then thoroughly washed using 0.01 phosphate buffer (pH 7.4), subsequently dehydrated in graded ethanol, toluene-cleared, and embedded in paraffin[16]. Histological quantification was performed in the testicular sections by assigning a Johnsen score as described previously[17]. Briefly, The Johnsen score which was based on the type of the cells damaged in the seminiferous tubules, was calculated by dividing the sum of all scores with the total number of seminiferous tubules examined.

Extraction of Spermatogenic Cells and Annexin V-propidium Iodide Assay

Extraction of spermatogenic cells assay was performed as described previously[18]. The Annexin-V FITC Apoptosis Detection Kit was used to implement apoptosis assay (Invitrogen, California, USA). The process was as follows: 100 mL spermatogenic cells were first suspended in 400 mL cold reaction buffer (HEPES; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) containing 2.5 mmol/L $CaCl_2$. Then the mixture was labeled with 10 mL annexin-V/fluorescein isothiocyanate solution and PI respectively for apoptotic cells detection under 15 min incubating at room temperature in the dark. And then cells were washed with 1 mL PBS, subsequently centrifuged, and resuspended in 300 mL reaction buffer. Finally, a FCM analysis was done to quantitatively determine the percentage of early or late stage apoptotic, necrotic, and viable cells. A total of 20 000 events were accumulated for each measurement.

Immunoblotting and Immunohistology

Testicular tissue was treated with a lysis buffer containing 7 mol/L urea, 2 mol/L thiourea, 4% (W/V) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propan e sulfonate (Chaps), and 2% (W/V) dithiothreitol (DTT) in the presence of 1% (V/W) protease inhibitor cocktail (Sigma Chemical, St. Louis, MO, USA). Protein concentration was measured by the Bio-Rad Bradford protein assay while bovine serum albumin (Sigma) was used as a standard. Protein extracted from the testis (60 μg/10 μL) was resolved in SDS-PAGE [12% (W/V) polyacrylamide gel] and then transferred to a PVDF membrane. The membrane was blocked with 5% skimmed milk in Tris buffer saline (TBS), and immunoblotted with the rabbit polyclonal IgG anti-p53 (Cat. #SC-6243), anti-PCNA (Cat. #SC-7907), anti-Bax (Cat. #SC-493) (Santa cruz, California, USA), anti-β-actin (Cat. #13E5) (Cell signal, Boston, USA), and a horseradish peroxidase-labeled secondary antibody (Cat. #ZB2301) (Shanghai Biyuntian Biotechnology Co., Shanghai, China). Immunoreactivity was detected using an enhanced chemiluminescent HRP substrate kit (Millipore, Billerrica, USA) and the images were captured by a FluorChem 2 imaging system (Alpha Innotech, San Leandro, CA, USA). Quantitative analysis of the relative density of the bands in Western blots was performed using the Quantity One 4.5.2 image analysis software (Bio-Rad Laboratories, USA). Images were corrected for background and expressed as optical density (OD/mm^2).

For immunohistochemistry, the testes fixed before were embedded in paraffin, sectioned at 5 μm, and mounted on silane-coated slides, subsequently dewaxed and rehydrated using alcohol and distilled water in sequence, followed by incubation in 2% hydrogen peroxide to quench endogenous peroxidase activity and then washed by PBS. Subsequently, they were blocked with goat serum (Beijing Boaoseng Biotechnology Co., Beijing, China) for 2 h and incubated with primary antibody (Santa cruz, California, USA; 1:500) overnight at 4 °C. After three times of washes using PBS, sections were incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit secondary antibody (Beijing Boaoseng Biotechnology) for 20 min at room temperature. Immunoreactive sites were visualized with diaminobezidine (DAB) and mounted for examination under bright field microscopy (Axio10, Zeiss, Germany).

Sperm Counts and Sperm Viability Analysis

For sperm sampling, the cauda epididymidis was placed in a pre-warmed petri dish containing 5 mL saline at 37 °C for 10 min to allow the sperm to swim up[19]. The 100 μL sperm suspension was mixed with 500 μL buffer (containing 0.01 mol/L Tris-HCl,

0.15 mol/L NaCl, and 0.001 mol/L ethylenediamine tetraacetic acid [EDTA], pH 7.4) and immediately placed into liquid nitrogen for sperm DNA damage assay[7]. The 1 mL sperm suspension was subsequently transferred into 2-mL microcentrifuge tubes, and sperm counts were conducted using a haemocytometer, under light microscope at ×200 (Nikon, Japan). Sperm viability was tested using MTT assay. The 50 μL sperm suspension was mixed with 25 μL MTT reagent and incubated in 96-well microplate at 37 °C for 2 h. After exhaustive centrifugation and pipetting, 100 μL DMSO was added to dissolve the formazan. Sperm activity was estimated at an absorbance of 490 nm (Tecan M200, Switzerland)[20].

Sperm DNA Damage Assay

Sperm DNA damage assay (SCSA) was carried out as described previously[5,7,21]. The sperm were analyzed by flow cytometry using FACScaliber (Becton Dickinson, San Jose, CA, USA). A total of 20 000 events were accumulated for each measurement. Acridine orange (AO, Sigma Chemical, St. Louis, MO, USA) was intercalated in double-stranded DNA emitting green fluorescence, and AO was associated with single-stranded DNA emitting red fluorescence. Sperm DNA damage was quantified by flow cytometry when the emission shifted from green to red fluorescence. %DFI = % of sperm outside the main population of a reference sample; and %HDS = % sperm with high green fluorescence due to lack of nuclear condensation. Using the areas defined as R1 (intact), R2 (highly fragmented), and R3 (not highly fragmented), the DFI was determined by the value of (R2+R3)/(R1+R2 +R3) and the HDS was determined by the value of R2/(R1+R2+R3).

Statistical Analysis

The results were expressed as mean±SEM. One-way ANOVA and Duncan's post hoc test were used to analyze the difference under the SPSS/PC software package environment (Version 19.0; SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered statistically significant.

RESULTS

Analysis of Caudal Sperm Count, Sperm Viability, Sperm DNA Damage, and Spermatogenic Cells Apoptosis Index

The caudal sperm count was observed to have obviously decline in the SUS group ($P<0.05$) and other experimental groups (SUS+0.25 Gy group, $P<0.01$; other groups, $P<0.001$) compared with the control group (Table 1) while the apoptosis index was detected to be significantly increasing in the SUS group ($P<0.05$) and other experimental groups ($P<0.001$) (Figure 1). The sperm viability was found to have changed little in the SUS group, but significantly reduced in the SUS + IR ($P<0.001$) and 2 Gy groups ($P<0.001$) (Table 1). The %HDS and %DFI increased significantly in the experimental groups compared with those in the control group ($P<0.001$) (Table 2, Figure 2).

Table 1. The Effect of Simulated Microgravity Conditions and CIR on Caudal Sperm Count and Viability

	Control	SUS	SUS+0.25 Gy	SUS+0.5 Gy	SUS+1 Gy	SUS+2 Gy	2 Gy
Epididymal Count (10^6)	8.94±0.8278	8.013±0.2013*	7.24±0.3175**	6.707±0.8317***	6.107±0.1665***	6.08±0.4***	5.087±0.3931***
Viability	0.0907±0.0093	0.0905±0.0054	0.0716±0.003***	0.071±0.0027***	0.0704±0.0021***	0.0689±0.0007***	0.0695±0.0004***

Note. Values represent the average ±S.E.M. *indicate a statistically significant difference: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ using one-way ANOVA and Duncan's post hoc analysis.

Table 2. The Effect of Simulated Microgravity Conditions and CIR on Sperm DNA Damage

	Control	SUS	SUS+0.25 Gy	SUS+0.5 Gy	SUS+1 Gy	SUS+2 Gy	2 Gy
HDS (%)	3.903±0.1721	6.22±0.1375***	7.31±0.36***	8.533±0.2516***	8.68±0.0854***	10.06±0.3412***	12.03±0.3523***
DFI (%)	9.547±0.3172	12.8±1.054***	13.5±0.071***	16.47±1.061***	16.67±0.4483***	18.41±0.1274***	21.35±0.7879***

Note. Values represent the average ±S.E.M. *indicate a statistically significant difference: $^{***}P<0.001$ using one-way ANOVA and Duncan's post hoc analysis.

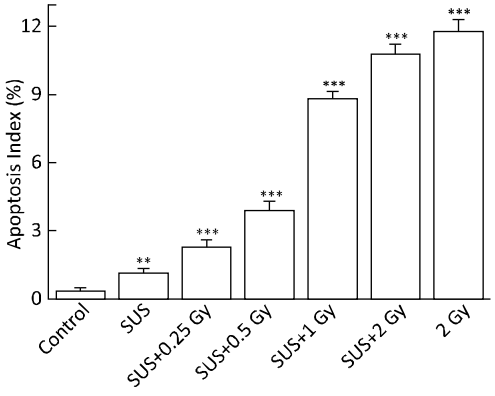

Figure 1. The effect of simulated microgravity conditions and CIR on apoptosis of spermatogenic cells. Values represent the means±S.E.M. * indicate a statistically significant difference: ** $P<0.01$, *** $P<0.001$ using one-way ANOVA and Duncan's post hoc analysis.

Testicular Histological Changes

A normal spermatogenesis, i.e. smooth seminiferous tubules, and regular spermatogenic cells, was identified in the control group and SUS group. Testis pathological changes were observed in the SUS+IR and 2 Gy groups and were seriously affected. Compared with the control group, a disordered arrangement of spermatogenic cells was observed, luminal narrowing was present, cytoplasmic vacuolization was noted, and the number of spermatids was smaller in the SUS+2 Gy group and 2 Gy group, respectively (Figure 3). The quantitative assessment of the seminiferous tubules was carried out based on the type of cells damaged, and the Johnsen score was assigned from 1 to 10 accordingly. A significant damage in the seminiferous tubule was observed in the SUS+IR groups and the IR group (Figure 4).

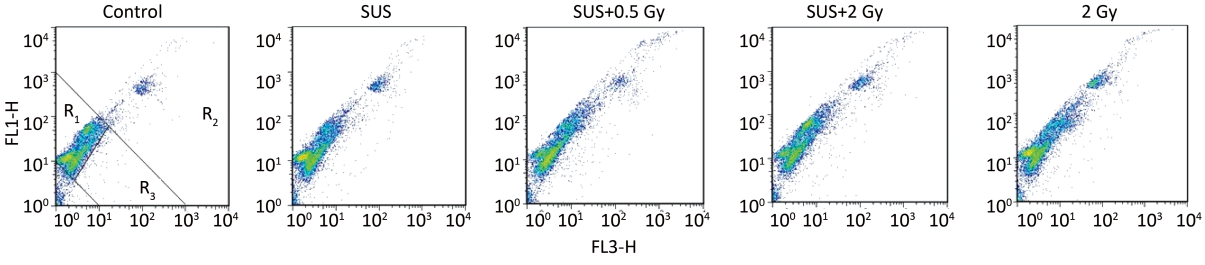

Figure 2. Sperm DNA damage quantified by flow cytometry.

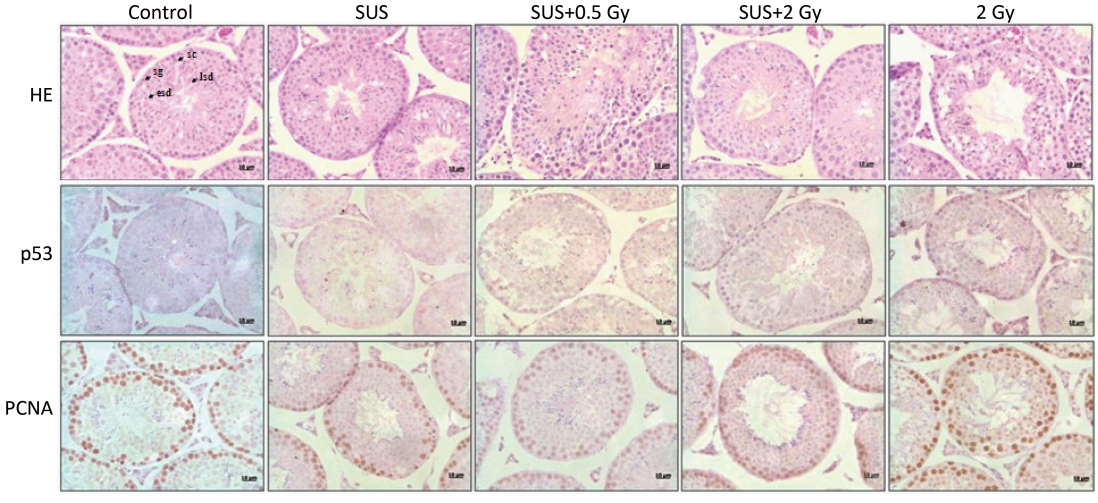

Figure 3. Photomicrographs of HE staining in testis sections and p53-, PCNA- positive expression in testis sections by immunohistology. sg, spermatogonia; sc, spermatocytes; esd, early spermatids; lsd, late spermatids. Dark brown is positive cells. Slides were performed at 40× magnification. Bar=10 μm.

Analysis of Immunohistology and Immunoblotting

The p53 expression level in the spermatogenic cells was almost zero, and a low stromal cell expression was observed in the control group. Positive p53 expression was witnessed in the spermatogonia and primary spermatocytes in the SUS group, but the increase was not significant compared with that in the control group. In the SUS+IR groups and the 2 Gy group, the positive p53 expression in spermatogenic cells indicated a significant increase. Particularly in the SUS+2 Gy and 2 Gy groups, the positive p53 expression of sperm cells and mesenchymal cells also changed significantly (Figure 3). The immunoblotting assay showed a significant increase of p53 protein expression in the SUS+IR (P<0.001) and 2 Gy groups (P<0.001) (Figure 5). A significant increase in Bax protein expression was observed in the experimental groups compared with that in the control group (P<0.001) (Figure 5). Positive PCNA expression of spermatogenic cells was observed in each group, mainly in the spermatogonia and spermatocytes. Low or no sperm cell expression was indicated (Figure 3). As shown in the immunoblotting assay, the PCNA expression in the SUS, SUS+0.25 Gy, and SUS+0.5 Gy groups decreased significantly (P<0.001), whereas it increased significantly (P<0.01) in the SUS+1 Gy group compared with that in the control group. Meanwhile, PCNA expression in the SUS+IR groups was elevated significantly (P<0.001) compared with that in the SUS group. However, PCNA expression declined significantly (P<0.05) in the 2 Gy group compared with that in the SUS+2 Gy group (Figure 5).

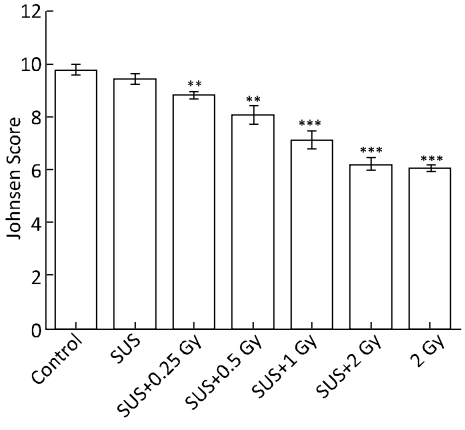

Figure 4. Testicular damage by Johnsen scoring. Values represent the means ±S.E.M. *indicate a statistically significant difference: $^{**}P$<0.01, $^{***}P$<0.001 using one-way ANOVA and Duncan's post hoc analysis.

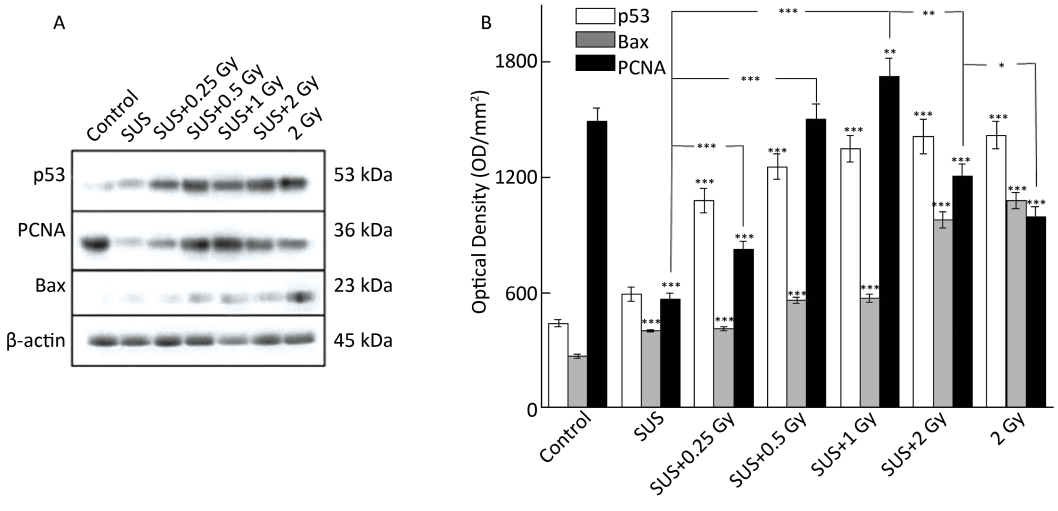

Figure 5. The expression levels of p53, PCNA and Bax protein were examined with Western blot assay (A). Relative expression of p53, PCNA and Bax protein (B). Values represent the means±S.E.M. *indicate a statistically significant difference: $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 using one-way ANOVA and Duncan's post hoc analysis.

DISCUSSION

The findings of the present study demonstrated that the SUS combined IR had a negative effect on spermatogenesis to cause spermatogenic cell apoptosis and proliferation imbalance. Spermatogenic cells apoptosis was found to have significantly increased in each experimental group. Meanwhile, SUS+IR increased spermatogenic cells apoptosis and disorder, thereby reducing sperm count and affecting sperm DNA integrity and viability.

Wang et al. studied the apoptotic effect of spermatogenic cells by CIR (0.1, 0.5, 1, 1.5, 2 Gy), and found that the apoptosis rate reached the peaks in 12 h after irradiation, and increased over time (12 h ago)[22]. However, excessive apoptosis of spermatogenic cells might induce spermatogenic disorders as one of the main reasons for male infertility[23]. Under normal circumstances, apoptosis and proliferation of spermatogenic cells maintain a dynamic equilibrium, thus regulating spermatogenesis and spermatogenic cells. Barriers to sperm from spermatogenic cells apoptosis and proliferation are believed to be caused by: (1) increased apoptosis and decreased proliferative capacity; (2) increased apoptosis and proliferation (more apoptosis than proliferation); (3) significantly inhibited proliferative capacity (although apoptosis was not enhanced); (4) slight change in spermatogenic cells apoptosis coupled with markedly inhibited proliferation[24].

Our data indicated that the SUS+IR increased spermatogenic cells apoptosis and enhanced proliferation. PCNA is a protein present in the nucleus, which represents the proliferative capacity of spermatogonia and primary spermatocytes[25]. The increase in PCNA expression levels may be induced by differentiation of spermatogonia and primary spermatocytes, and induced spermatogenic cells apoptosis. In the present study, the PCNA expression in histology indicated that only spermatogonia and primary spermatocytes had DNA synthesis, and SUS could inhibit the proliferation of spermatogenic cells. The tendency of p53 and Bax expression was similar to that of PCNA under SUS. Compared with the SUS+1 Gy group, the SUS+2 Gy group exhibited a significant reduction in PCNA and increased p53 and Bax expressions, indicating that irradiation overdose caused an irreversible damage to the testis and the testis was consequently unable to increase PCNA expression for proliferation balance.

p53 expression in the five groups (from the SUS group to the SUS+2 Gy group) increased with doses, indicating that SUS+IR could aggravate spermatogenic cells apoptosis, and had a dose-effect relationship. The data of Bax expression tests confirmed the conclusion because Bax is an important apoptosis regulatory protein. Bax expression increased the apoptosis and survival imbalance leading to apoptosis. Recent studies suggested that Bax was involved in the process of spermatogenic cell apoptosis[26] and wild-type p53 might induce Bax expression[27]. The present study also revealed that spermatogenic cells apoptosis and sperm DNA damage in the SUS+2 Gy group were lower than those in the 2 Gy group, suggesting that SUS might resist radiation-induced spermatogenic cells apoptosis and sperm DNA damage. Nevertheless, the underlying mechanism remains unclear, which warrants further clarification. However, in the present study, the 2 Gy group showed higher p53 and Bax expression compared with the SUS+2 Gy group because PCNA expression in the SUS+2 Gy group was higher than that in the 2 Gy group. Therefore, the SUS+2 Gy group had a higher resistance to radiation than the 2 Gy group.

In general, the data obtained in the present study are in agreement with those of several authors who evaluated the expression of p53, Bax, PCNA related to cell apoptosis and proliferation. p53 protein plays a key role in the cell proliferation and apoptosis. On the one hand, as revealed by the present study, the overexpression of PCNA and p53 was present from SUS+0.25 Gy group to the SUS+1 Gy group, which was associated with the p53-mediated regulation of PCNA expression[28]. On the other hand, the expression of Bax was up regulated, because the p53 protein up regulated the Bax gene, and an over-expression of Bax would promote apoptosis and inhibit the anti-apoptotic activity pathway[27,29]. These two effects led to spermatogenic cells apoptosis and proliferation imbalance in simulated microgravity conditions and CIR.

Studies have shown that sperm DNA damage have a potential negative impact on male fertility[30]. Through a variety of detection methods, DNA damage in infertile males was found to be significantly higher than that in fertile males[31]. The association between DFI and HDS sperm abnormalities and male fertility has been confirmed

in several studies[32-34]. The results of this study indicate that sperm DNA damage induced by SUS+IR can also affect the male reproductive system. Meanwhile, sperm viability and sperm count are closely related to relevance and sensitivity. Higher HDS and DFI values, more inactive sperm and less sperm count indicate that the SUS+IR can cause sperm DNA damage and DNA fragmentation, with affecting the sperm viability. SCSA could be an objective analysis of sperm DNA integrity, allowing rapid identification and evaluation of sperm cells, thereby showing high sensitivity to DNA denaturation[35]. The sperm DNA damage as identified in the present study can be interpreted as SUS+IR could disturb spermatogenic cells proliferation and apoptosis balance, resulting in sperm chromatin packaging blockage by the apoptosis of spermatogenic cells and thereby causing sperm DNA damage. Direct sperm DNA damage may also result in SUS+IR. Two super-imposed effects have exhibited high HDS and DFI values.

In conclusion, simulated microgravity conditions and CIR can induce spermatogenic cells apoptosis and sperm DNA damage. The findings in our study provided evidence that SUS+IR could interfere with spermatogenesis: spermatogenic cells apoptosis and proliferation imbalance. Sperm DNA damage may be one of the underlying mechanisms leading to the decline in the fertility of males under microgravity conditions and space environment. Meanwhile, this study also laid down a scientific basis for the protection of the health and safety of astronauts and space travelers.

REFERENCE

1. Niu ZH, Shi HJ, Zhang HQ, et al. Sperm chromatin structure assay results after swim-up are related only to embryo quality but not to fertilization and pregnancy rates following IVF. Asian J Androl, 2011; 13, 862-6.
2. Mahfouz RZ, Sharma RK, Said TM, et al. Association of sperm apoptosis and DNA ploidy with sperm chromatin quality in human spermatozoa. Fertil and Steril, 2009; 91, 1110-8.
3. Bungum M, Bungum L, Giwercman A. Sperm chromatin structure assay (SCSA): a tool in diagnosis and treatment of infertility. Asian J Androl, 2011; 13, 69-75.
4. Hossain S, Johannisson A, Wallgren M, et al. Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art. Asian J Androl, 2011; 13, 406-19.
5. Evenson DP, Wixonb R. Environmental toxicants cause sperm DNA fragmentation as detected by the Sperm Chromatin Structure Assay (SCSA). Toxicol Appl Pharam, 2005; 207, S532-7.
6. Virro MR, Larson-Cook KL, Evenson DP. Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in *in vitro* fertilization and intracytoplasmic sperm injection cycles. Fertil and Steril, 2004; 81, 1289-95.
7. Kawase Y, Wada NA, Jishage K. Evaluation of DNA fragmentation of freeze-dried mouse sperm using a modified sperm chromatin structure assay. Theriogenology, 2009; 72, 1047-53.
8. Lugg DJ. Behavoiral health in Antarctcia: implications for long-duration space missions. Avait Space Environ Med, 2005; 76, B74-7.
9. Sharma CS, Sarkar S, Periyakaruppan A, et al. Single-walled carbon nanotubes induces oxidative stress in rat lung epithelial cells. J Nanosci Nanotechnol, 2007; 7, 2466-72.
10. Amann RP, Deaver DR, Zirkin BR, et al. Effect of microgravity or smiulated launch on testicular function in rat. J Appl Phisio, 1992; 72, 174-85.
11. Liu Y, Long J, Zhang LW, et al. Low-dose carbon ion irradiation effects on DNA damage and oxidative stress in the mouse testis. Adv Space Res, 2011; 47, 55-9.
12. Frigeri A, Iacobas DA, Iacobas S, et al. Effect of microgravity on gene expression in mouse brain. Exp Brain Res, 2008; 191, 289-300.
13. Bu G, Shuang F, Wu Y, et al. AQP9: A novel target for bone loss induced by microgravity. Biochem Biophys Res Commun, 2012; 419, 774-8.
14. Sharma CS, Sarkar S, Periyakaruppan A, et al. Simulated microgravity activates apoptosis and NF-κB in mice testis. Mol Cell Biochem, 2008; 313, 71-8.
15. Li Q, Dai Z, Yan Z, et al. Heavy-ion conformal irradiation in the shallow-seated tumor therapy terminal at HIRFL. Med Biol Eng Comput, 2007; 45, 1037-43.
16. Heechul K, Changjong M, Taekyun S. Immunohistochemical study of flotillin-1 in the rat testis during postnatal development. Acta histochemica, 2008; 110, 224-31.
17. Trivedi PP, Kushwaha S, Tripathi DN, et al. Evaluation of male germ cell toxicity in rats: Correlation between sperm head morphology and sperm comet assay. Mutation Research, 2010; 703, 115-21.
18. Sarabia L, Maurer I, Bustos-Obrego´n E. Melatonin prevents damage elicited by the organophosphorous pesticide diazinon on mouse sperm DNA. Ecotox Environl Safe, 2009; 72, 663-8.
19. Ma YX, Ebine N, Aoki K, et al. Effects of Cow's Milk on Reproduction in ICR Male Mice. Biomed Environ Sci, 2009; 22, 16-3.
20. Li HY, Zhang H. Proteome analysis for profiling infertility markers in male mouse sperm after carbon ion radiation. Toxicology, 2013; 306, 85-92.
21. Haines GA, Hendry JH, Daniel CP, et al. Increased levels of comet-detected spermatozoa DNA damage following *in vivo* isotopic- or X-irradiation of spermatogonia. Mutat Res, 2001; 495, 21-32.
22. Wang B, Murakami M, Eguchi-Kasai K, et al. Effects of prenatal irradiation with accelerated heavy-ion beams on postnatal development in rats: III. Testicular development and breeding activity. Adv Space Res, 2007; 40, 550-62.
23. Lin WW, Lamb DJ, Lipshultz LI, et al. Demonstration of testicular apoptosis in human male infertility states using a DNA laddering technique. Int Urol Nephrol, 1999; 31, 361-70.
24. Lin WW, Lamb DJ, Wheeler TM, et al. Apoptotic frequency is increased in spermatogenic maturation arrest and hypospermatogenic states. J Urol, 1997; 158, 1791-3.
25. Stger K, Aleithe I, Bethe H, et al. The proliferation of spermatogonia in normal and pathological human seminiferous epithelium: an immunohistochemical study using monoclonal antibodies against Ki-67 protein and proliferating

cell nuclear antigen. Mol Hum Reprod, 1998; 4, 227-33.
26.Saikumar P, Dong Z, Weinberg JM, et al. Mechanisms of cell death in hypoxia reoxygenation injury. Oncogene, 1998; 17, 3341-7.
27.Li HL, Huang XP, Zhou XH, et al. Correlation of seven biological factors (Hsp90a, p53, MDM2, Bcl-2, Bax, Cytochrome C, and Cleaved caspase3) with clinical outcomes of ALK+ anaplastic large-cell lymphoma. Biomed Environ Sci, 2011; 24, 630-41.
28.Xu J, Morris GF. p53-mediated regulation of proliferating cell nuclear antigen expression in cells exposed to ionizing radiation. Mol Cell Biol, 1999; 19, 12-20.
29.Ioachim E, Malamou-Mitsi V, Kamina SA, et al. Immunohistochemical expression of Bcl-2 protein in breast lesions: Correlation with Bax, p53, Rb, C-erbB-2, EGFR and proliferation indices. Anticancer Res, 2000; 20, 4221-6.
30.Pe´rez-Cerezales S, Miranda A, Gutie´rrez-Ada´n A. Comparison of four methods to evaluate sperm DNA integrity between mouse caput and cauda epididymidis. Asian J Androl, 2012; 14, 335-7.
31.Serqerie M, Laforest G, Bujan L, et al. Sperm DNA fragmentation: threshold value in male fertility. Hum Reprod, 2005; 20, 3446-51.
32.Lin MH, Kuo-Kuang Lee R, Li SH, et al. Sperm chromatin structure assay parameters are not related to fertilization rates, embryo quality, and pregnancy rates inin vitrofertilization and intracytoplasmic sperm injection, but might be related to spontaneous abortion rates. Fertil and Steril, 2008; 90, 352-9.
33.Virro MR, Larson-Cook KL, Evenson DP. Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. Fertil and Steril, 2004; 81, 1289-95.
34.Boe-Hanson GB, Fedder J, Ersboll AK, et al. The sperm chromatin structure assay as a diagnostic tool in the human fertility clinic. Hum Reprod, 2006; 21, 1576-82.
35.Matsuura R, Takeuchi T, Yoshida A. Preparation and incubation conditions affect the DNA integrity of ejaculated human spermatozoa. Asian J Androl, 2010; 12, 753-9.