Risk of simulated microgravity on testicular injury induced by high-LET carbon-ion beams in mice

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Abstract This study investigated the impact of simulated microgravity on acute injury induced by low doses of carbon ions in male reproductive organs of mice, and determined alterations in spermatogenic function and expression levels of apoptotic factors in mice following exposure to acute irradiation after 7 days of simulated microgravity. The results demonstrated that significant reductions in spermatozoa, primary spermatocytes and spermatogonia, and increased globular cells in seminiferous tubule and pro-apoptotic proteins were observed in the group exposed to over 0.4 Gy irradiation. Collectively, the data suggest that lesions inflicted by simulated microgravity are not markedly modified by lower doses of irradiation (0.2 Gy) in mouse testis compared to the control group. However, testicular impairments were markedly evident in the group exposed to higher doses of carbon ions plus simulated microgravity, which may be due at least in part to elevated apoptosis initiated by the mitochondrial apoptosis pathway in germ cells.

Key words Stimulated microgravity, High-LET irradiation, Carbon ion, Mouse testis

1 Introduction

Microgravity and space radiation are principal concerns for manned spaceflight, and serve as potentially limiting factors for interplanetary missions[1]. It is well-known that exposure to microgravity causes many negative effects on the body, such as cardiovascular and blood flow[2,3], bone metabolism[4], skeletal muscle atrophy[5], endocrine disturbances[6], immune responses[7], and embryogenesis[8]. Some previous reports have demonstrated that microgravity also results in alteration in spermatogenesis[9], pathological disorders[10], hormonal regulation[11] including testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH), and blood-testis barrier[12].

Space radiation is comprised of particles trapped in the Earth's magnetic field; particles are shot into space during solar flares and galactic cosmic rays, which are comprised of high-energy protons and heavy ions from outside of the solar system[13]. Heavy ions are relatively rare, but may exhibit high-LET (liner energy transfer) values that produce more irreparable DNA breaks[14] and chromosomal aberrations[15]; thus, high-LET radiation is more cytotoxic and genotoxic to cells[16,17]. The testis is one of the most radiosensitive organs of the body. Our previous findings demonstrated that heavy ions can lead to prominent morphological damage[18], spermatogenesis obstruction[19], and destruction of poly(ADP-ribose) polymerase (PARP) activity and its expression, which is linked with DNA repair[20] and increased sper-matocyte chromosomal aberrations[21] in mouse testis.

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With the expansion of human space missions, crew members of child-bearing age are increasingly concerned about the potential risk to the offspring. Thus, a more reliable estimation of the microgravity environment and high-LET radiation is warranted.

Based on the ground experiments at a heavy ion accelerator and a tail-suspension model, the aim of this study was to evaluate the combined influence of simulated microgravity for 7 days and subsequent irradiation with low-dose carbon ion beams on outbred Kunming strain mouse testis.

2 Materials and methods

2.1 Animals

Male mice (30–35 g) of outbred Kunming strain obtained from Lanzhou Medical College (Lanzhou, China) were used in this study. All animal studies were performed according to the requirements of the Animal Care Committee at the Institute. Mice were maintained at a constant temperature ((22±1)°C) with 12 h light and dark cycles.

2.2 Irradiation procedure

A mouse was positioned in a chamber fixed to the irradiation equipment at the Heavy Ion Research Facility in Lanzhou (HIRFL, Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou, China). The whole body of the mouse was irradiated with carbon ion beams at energy 235 $MeV \cdot u^{-1}$ and LET 29.6 $keV \cdot \mu m^{-1}$ in the water (plateau region) generated from HIRFL, with a dose rate approximately 1.0 $Gy \cdot min^{-1}$. The collimation of the beams to the site of irradiation was controlled by a microcomputer. The acquisition of data (preset numbers converted by doses of irradiation) was automatically performed using a microcomputer during irradiation. Particle fluence rate was determined using air ionizing chamber signal according to the calibration of the detector (Type: PTW-UNIDOS, PTW-FREIBURG Co., Germany). Dose was calculated by particle fluence rate and LET.

2.3 Simulated microgravity: tail suspension (TL)

Tail suspension technique was performed according to the design described by Chen *et al.*[22] and Kamiya *et al.*[9] to simulate microgravity. Briefly, a strip of orthopedic tape was applied to the mouse tail and fastened to an overhead suspension bar. Next, the mounted mice were adjusted to a position in which the head was tilted down approximately 30^{o} and the hind legs did not touch the floor of the housing unit. The mice were subjected to TL for 7 days. Meanwhile, control mice were housed individually in similar conditions, but with hind legs on the floor.

2.4 Sample collections

A total of 48 animals were randomly divided into six groups each containing 8 individuals. The first group was no radiation or simulated microgravity treatment as control group. The second group was only received tail suspension for 7 days as microgravity -treated group (TL group). The rest four groups were continuously administrated modeled microgravity for 7 days prior to irradiation, and subsequently were exposed to 0.2 Gy, 0.4 Gy, 0.8 Gy, and 1.0 Gy of carbon-ion beams, respectively. After this, the animals were sacrificed by cervical dislocation 24h after irradiation. Testes were quickly removed, and then fresh samples were used for analysis of spermatogenic function analysis, and residual samples immediately were frozen and stored at –80°C until other biochemical determinations.

2.5 Sperm count and morphology

The caudal epididymis samples were placed in 4ml sodium chloride solution (0.86%) and minced finely using small scissors. The suspensions were dispersed and filtered to exclude large tissue fragments. Eosin Y (1%) was added for staining sperm morphology. Sperm counts and sperm abnormalities were recorded under light microscope. Abnormal forms of sperm were classified into head morphological abnormalities: amorphous heads and two heads.

2.6 Quantification of testicular spermatogenesis by histological evaluation

After sacrificing the animals by cervical dislocation, the left testis was extracted and fixed in 10% buffered formalin solution immediately. Testis tissue samples

were then dehydrated in graded ethanol, toluene-cleared, and embedded in paraffin. Each section is 5μm thick and stained with hematoxylin and eosin (H & E), and examined using light microscope (Olympus). A total of 20 round seminiferous tubules were randomly selected in 10 HE-stained slides (20×10= 200 seminiferous tubules in each groups), and the numbers of spermatogonia or primary spermatocytes per tubule were counted.

2.7 Western blot analysis

The testes were washed with PBS and then homogenized in 1 mL radioimmunoprecipitation (RIPA) buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, pH 8.0, 1mM EGTA, 0.5% Deoxycholate) with 100μg/mL phenylmethylsulfonyl fluoride followed by centrifugation at 12 000×g for 20 min. The supernatant was collected and the amount of protein was estimated using BCA protein assay kit. Protein samples were loaded onto 10% sodium dodesyl sulfate (SDS)-polyacrylamide gel, separated, and then transferred to polyvinylidine difluoride (PVDF) membranes (Millipore Corporation, USA). The membrane was blocked and subsequently incubated with anti-Bax antibody (Santa Cruz Biotechnology, Inc.), anti-Bcl-xL antibody (Santa Cruz Biotechnology, Inc), anti-caspase-3 antibody (Bioworld Technology Inc), and anti-β-actin antibody (Santa Cruz Biotechnology, Inc.) overnight at 4°C. Following primary antibody incubation, the membranes were washed three times for 5 min each with TBST and incubated in HRP-conjugated secondary antibody. Secondary antibody probes were detected using ECL Western blot detection reagents (GE Healthcare). The expression levels of proteins were quantified using FluorChem FC2 software (Alpha Innotech Corporation).

2.8 Statistical analysis

The results were expressed as mean ± standard error (SEM). Multiple comparisons were performed using one-way ANOVA followed by LSD as a post-hoc test. Statistical differences between the two groups were analyzed using the Student's t-test. A *p*-value of less than 0.05 was selected as a criterion for a statistically significant difference.

3 Results

3.1 Effects of microgravity on sperm count induced by carbon-ion irradiation

The sperm abnormal sperm numbers of treatment groups as a ratio of control group are given in Fig.1. In comparison with the control group, 22.3%, 21.2% or 31.5% reduction of sperm number was found in the TL plus 0.4 Gy, 0.8 Gy and 1.0 Gy group. The induction of sperm head abnormalities is a very sensitive assay for well establishing the response of reproductive tissue to ionizing radiations[23]. In our study, a significant enhancement in the number of abnormal spermatozoa was observed in the TL group or TL plus irradiated group (P<0.01) compared to the control group, but there is no radiation dose-dependent response, indicating that the sperm malformation could be mainly attribute to the effect of microgravity.

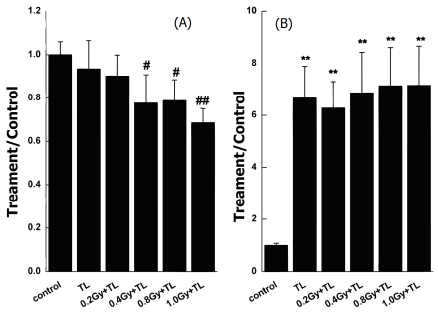

Fig.1 Effects of microgravity on sperm count and sperm abnormalty induced by carbon-ion irradiation. Mice were treated with tail suspension and whole body exposure to carbon ions, and then sacrificed 24 h after irradiation. Sperm damage was assessed according to the number of spermatozoa, expressed as mean±SEM of 8 mice per group. $^{\#}P$ <0.05, $^{\#\#}P$ <0.01 (Spermatozoa), and $^{**}P$<0.01 (Abnormal Spermatozoa) vs. control group for the treated groups, respectively.

3.2 Effects of microgravity on histopathological level induced by carbon-ion irradiation

To evaluate the alterations in spermatogenesis function following exposure to ionizing radiation and simulated microgravity, numbers of spermatogonia or primary spermatocytes per seminiferous tubule of treatment groups as a ratio of control group were quantitatively shown in Fig.2A. The average values of spermatogonia receiving 0.4 Gy (P<0.05), 0.8 Gy (P<0.01), and 1.0 Gy (P<0.001) irradiation plus simulated

microgravity treatment were significantly lower compared to the control group. In particular, the spermatogonia of 1.0 Gy irradiated plus TL-treatment group was decreased by 39.4% compared to the control group. And the numbers of primary spermatocytes induced by the combined effects remarkbally reduced in over 0.4 Gy irradiation group (P<0.05). The photomicrographs in Fig.2B illustrate the abnormal globular (multinucleated) cells in the over 0.4 Gy irradiated treatment groups and differing distribution of spermatogenic epithelium (a: Spermatocytes, b: Spermatids).

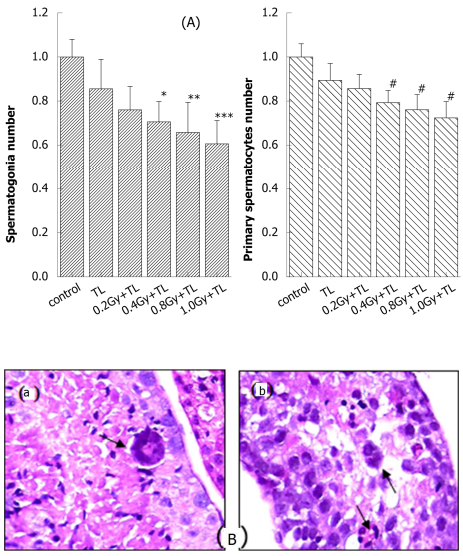

Fig.2 Effects of microgravity on the histopathological level induced by carbon-ion irradiation. Mice were subjected to tail suspension and whole body exposure to carbon ions, and then sacrificed 24h after irradiation. (A) Spermatogenesis function was assessed according to the number of spermatogonia and primary spermatocytes per tubule expressed as mean ± SEM of 8 animals per group. *P<0.05, **P<0.01, ***P<0.001 (spermatogonia) and $^{\#}P$<0.05 (primary spermatocytes) vs. control group for the treated groups. (B) Typical photomicrographs (original magnification: 400×) of multinucleated cells in the seminiferous tubules when animals were administrated simulated microgravity combined with carbon ion- irradiation; black block arrows indicate the globular (multinucleated) cells.

3.3 Effects of microgravity on the expression of apoptotic protein Bcl-2 family induced by carbon-ion irradiation

To further characterize the molecular mechanisms underlying apoptosis induced by carbon ion irradiation under simulated microgravity, we analyzed apoptosis regulatory proteins using Western blot analysis. The Bcl-2 family involves anti-apoptotic members such as Bcl-xL, and pro-apoptotic members such as Bax. The balance of Bax/Bcl-xL ratio determines the germ cells to cell death or survival following simulated microgravity and radiation. Fig.3A shows that over 0.8 Gy of irradiation plus microgravity groups induced a notable decrease in the protein level of Bcl-xL compared to the control group. There were approximately 1.58 and 1.62-fold reductions in relative Bax/Bcl-xL expression in the control group compared with 0.8 Gy and 1.0 Gy irradiated plus TL-treatment group.

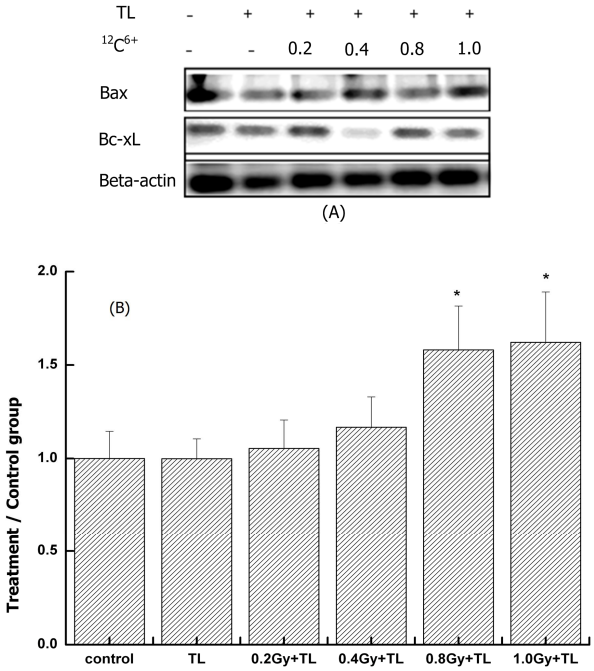

Fig.3 Effects of microgravity on the expression of apoptotic protein induced by carbon-ion irradiation. (A) Assessment of Bax and Bcl-xL protein in testis tissues by Western blot analysis. (B) The values of Bax /Bcl-xL ratio are expressed as mean± SEM of 8 animals per group. *P<0.05 vs. control group for the other treated groups.

3.4 Effects of microgravity on the expression of caspase-3 caused by carbon-ion irradiation

Caspase-3 acts as the major executors of apoptosis. As can be seen in Fig.4, activation of caspase-3 was markedly evident in mouse testis following irradiation with 0.4 Gy (P<0.05), 0.8 Gy (P<0.01) as well as 1.0 Gy (P<0.01) irradiated plus TL-treatment group compared to the control group.

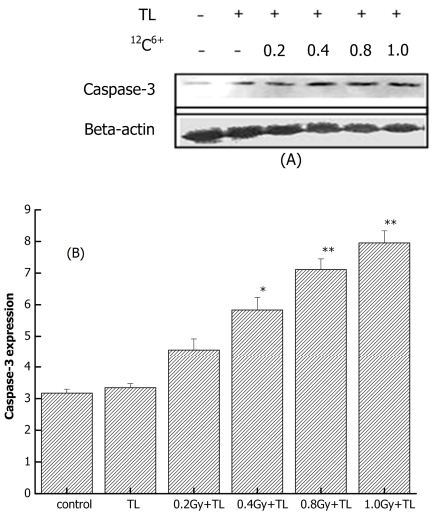

Fig.4. Influence of microgravity on the expression of apoptotic protein induced by carbon-ion irradiation. (A) Evaluation of caspase-3 protein in testis tissues by Western blot analysis. (B) The values of caspase-3 are expressed as mean± SEM of 8 animals per group (caspase-3/β-actin). **P* <0.05, ***P* <0.01 vs. control group for the other treated groups.

4 Discussion

The interplay between microgravity and space radiation potentially poses a great hazard to humans in space. In this study, we explored the combined influence of simulated microgravity on the male reproductive system using a tail-suspension model and high-LET carbon ion irradiation produced by heavy ion accelerators.

After 7 days of TL and subsequent carbon ion-irradiation, numbers of spermatozoa or spermatogonia were highly reduced according to H&E staining over 0.4 Gy irradiation and microgravity groups compared to the control group; this finding demonstrated that the spermatogenesis process can potentially be blocked. However, there were no statistically significant differences in TL-treated mice and lower dose irradiation combined TL-treated mice. We thought that tail or hind-limb suspension of a short duration may change serum hormonal levels such as testosterone (data not Shown), FSH, and LH in Leydig cells[11], as well as sperm abnormality (Fig.1), but may fail to cause a notable decrease in spermatogenetic cells of spermatogonia or primary spermatocytes. Hence, the deleterious spermatogenesis in our study may be attributed to the acute effects of high-LET irradiation. Our previous data also reported that male germ cells irradiated with over 0.5 Gy of carbon ion may exhibit chronic or delayed damage[19]. Furthermore, it is worthy to note that a proportion of sperm was partly but not significantly decreased in the TL and lower dose irradiation treatment groups, which indicates that the modeled microgravity did not markedly interfere with the increased damage of seminiferous tubules induced by carbon-ion irradiation.

The results from H&E staining showed that globular (multinucleated) cells occur in over 0.4 Gy irradiation treatment groups with differing distributions of spermatogenic epithelium. Multinuclear germ cell can lead to impairment of spermatogenesis and even infertility[24]. The formation of these multinuclear cells can most likely be explained by the action of simulated microgravity and high-LET irradiation on the prolonged process of meiotic division in germ cells, which is similar to the effect of boric acid on seminiferous tubules[25]. Previously, Forsman *et al.*[26] reported seeing multinucleated cells in seminiferous tubules of rats exposed to antiothoastatic suspension.

The killing of male germ cells by radiation and other toxicants has been previously attributed to apoptosis[27]. Normally, apoptosis of germ cells is required for spermatogenesis and homeostasis; it can remove abnormal or superfluous cells at specific checkpoints, establish caste differentiation, and individualize gametes[28]. Our data previously reported indicated that carbon ion irradiation induced a dose-dependant increase in the percentage of apoptotic index under simulated microgravity conditions, and that the apoptotic cell values of 1.0 Gy group exhibited a 3.16-fold increase compared to the control group[29]. To further understand how irradiation in cooperation with microgravity regulates the mitochondrial apoptosis pathway in mouse testis, the relative levels of proapoptotic vs antiapoptotic Bcl-2 family members were determined in all treatment groups 24 h after irradiation. The data indicated that over 0.8 Gy of irradiation plus microgravity induced a notable up-regulation of Bax/Bcl-xL ratio, suggesting that the treatments promoted apoptosis in germ cells. Zou *et al.*[30] also found that microgravity induced leukemic cell apoptosis through regulation of Bcl-2 family proteins. The marked increase of caspase-3 activity in over 0.4 Gy irradiation plus microgravity groups may

be involved in the execution of apoptosis as an underlying mechanism of radiation-induced germ cell loss. Currently, little information is available concerning the relationship between radiation and microgravity. Further investigation of the interactions between high-LET irradiation and simulated microgravity is warranted.

In conclusion, the present study primarily demonstrated that microgravity plus lower doses of irradiation treatment may not harm the testes, whereas higher dose irradiation (0.4–1.0 Gy) combined with microgravity treatments elicited significant impairment in mouse testes compared to the control group and may be responsible, at least in part, for initiation of the mitochondrial apoptosis pathway in germ cells.

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