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

Authors: LIU Yang, ZHANG Luwei, ZHANG Hong, WU Zhenhua, Wang Zhenhua, WANG Yibo, LI Hongyan, MA Xiaofei, Xie Yi

Date: 2023-06-18T00:00:00+00:00

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.

Full Text

Preamble

Risk of Simulated Microgravity on Testicular Injury Induced by High-LET Carbon-Ion Beams in Mice

Authors: LIU Yang[1,2,3], ZHANG Luwei[1,2,3], ZHANG Hong[1,2,3,*], WU Zhenhua[1,2,3], WANG Zhenhua[1,2,3], WANG Yibo[4], LI Hongyan[1,2,3], MA Xiaofei[1,2,3], XIE Yi[1,2,3]

Affiliations: [1]Department of Heavy Ion Radiation Medicine, Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou 730000, China [2]Key Labo-

ratory of Heavy Ion Radiation Biology and Medicine of Chinese Academy of Sciences, Lanzhou 730000, China [3]Key Laboratory of Heavy Ion Radiation Medicine of Gansu Province, Lanzhou 730000, China [4]College of Life Science and Chemistry, Tianshui Normal University, Tianshui 741000, China

Abstract: This study investigated the impact of simulated microgravity on acute injury induced by low doses of carbon ions in the male reproductive organs of mice, and determined alterations in spermatogenic function and expression levels of apoptotic factors following exposure to acute irradiation after 7 days of simulated microgravity. The results demonstrated significant reductions in spermatozoa, primary spermatocytes, and spermatogonia, along with increased globular cells in seminiferous tubules and elevated pro-apoptotic proteins in groups 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 groups 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.

Keywords: Simulated microgravity, High-LET irradiation, Carbon ion, Mouse testis

Introduction

It is well established that microgravity and space radiation are principal concerns for manned spaceflight and serve as potentially limiting factors for interplanetary missions [1]. Microgravity causes numerous adverse effects on the body, including cardiovascular and blood flow alterations [2,3], bone metabolism disruption [4], skeletal muscle atrophy [5], endocrine disturbances [6], and impaired embryogenesis [8]. Previous reports have demonstrated that microgravity also disrupts spermatogenesis [9], hormonal regulation [11], and immune responses [7]. Specifically, microgravity induces pathological alterations in testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and the blood-testis barrier [10,12].

Space radiation comprises particles trapped in the Earth's magnetic field, solar flare emissions, and galactic cosmic rays consisting of high-energy protons and heavy ions from outside the solar system [13]. Heavy ions are relatively rare but exhibit high linear energy transfer (LET) 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 in the body. Our previous findings demonstrated that heavy ions can cause prominent damage [18], spermatogenesis obstruction [19], and destruction of poly(ADP-ribose) polymerase (PARP) activity and expression, which is linked with DNA repair [20] and increased spermatocyte chromosomal aberrations [21] in mouse testis.

With the expansion of human space missions, crew members of child-bearing age are increasingly concerned about potential risks to their offspring. Thus, a more reliable estimation of the risks posed by the microgravity environment and high-LET radiation is warranted. Based on ground experiments using a heavy ion accelerator and a tail-suspension model, this study aimed to evaluate the combined influence of 7 days of simulated microgravity and subsequent low-dose carbon ion beam irradiation on the testes of outbred Kunming strain mice.

2. Materials and Methods

2.1 Animals

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

2.2 Irradiation Procedure

Each 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 each mouse was irradiated with carbon ion beams at an energy of 235 MeV $\cdot$ u^{-1} and LET of 29.6 keV $\cdot$ m^{-1} in water (plateau region) generated from HIRFL, with a dose rate of approximately 1.0 Gy $\cdot$ min^{-1}. Beam collimation was controlled by a microcomputer, and data acquisition (preset numbers converted by irradiation doses) was performed automatically during irradiation. Particle fluence rate was determined using an air ionization chamber signal according to detector calibration (Type: PTW-UNIDOS, PTW-FREIBURG Co., Germany). Dose was calculated from particle fluence rate and LET.

2.3 Simulated Microgravity: Tail Suspension (TL)

Tail suspension was performed according to methods 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. The mounted mice were adjusted to a position in which the head was tilted down approximately 30° and the hind legs did not touch the floor of the housing unit. Mice were subjected to tail suspension for 7 days. Control mice were housed individually under similar conditions but with hind legs on the floor.

2.4 Sample Collection

A total of 48 animals were randomly divided into six groups of 8 individuals each. The first group received no radiation or simulated microgravity treatment and served as the control group. The second group received only tail suspension

for 7 days as the microgravity-treated group (TL group). The remaining four groups were administered simulated microgravity for 7 days prior to irradiation and subsequently exposed to 0.2 Gy, 0.4 Gy, 0.8 Gy, and 1.0 Gy of carbon-ion beams, respectively. Animals were sacrificed by cervical dislocation 24 hours after irradiation. Testes were quickly removed; fresh samples were used for spermatogenic function analysis, and residual samples were immediately frozen and stored at −80°C for subsequent biochemical determinations.

2.5 Sperm Count and Morphology

Caudal epididymis samples were placed in 4 mL 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 sperm morphology staining. Sperm counts and abnormalities were recorded under a light microscope. Abnormal sperm forms were classified as head morphological abnormalities, including amorphous heads and two heads.

2.6 Quantification of Testicular Spermatogenesis by Histological Evaluation

After sacrificing animals by cervical dislocation, the left testis was extracted and immediately fixed in 10% buffered formalin solution. Testis tissue samples were then dehydrated in graded ethanol, cleared in toluene, and embedded in paraffin. Each section was cut to 5 m thickness, stained with hematoxylin and eosin (H&E), and examined using a light microscope (Olympus). A total of 20 round seminiferous tubules were randomly selected from 10 H&E-stained slides (20$×$10 = 200 seminiferous tubules per group), and the numbers of spermatogonia or primary spermatocytes per tubule were counted.

2.7 Western Blot Analysis

Testes were washed with PBS and homogenized in 1 mL radioimmunoprecipitation (RIPA) buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, pH 8.0, 1 mM EGTA, 0.5% deoxycholate) with 100 µg/mL phenylmethylsulfonyl fluoride, followed by centrifugation at 12,000×g for 20 min. The supernatant was collected and protein concentration was estimated using a BCA protein assay kit. Protein samples were loaded onto 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels, separated, and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, USA). The membrane was blocked and subsequently incubated overnight at 4°C 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.). Following primary antibody incubation, membranes were washed three times for 5 min each with TBST and incubated with HRP-conjugated secondary antibody. Secondary antibody probes were detected using ECL Western blot detection reagents (GE Healthcare). Pro-

tein expression levels were quantified using FluorChem FC2 software (Alpha Innotech Corporation).

2.8 Statistical Analysis

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

3. Results

3.1 Effects of Microgravity on Sperm Count and Morphology Induced by Carbon-Ion Irradiation

Sperm numbers and abnormal sperm counts in treatment groups are presented as ratios of the control group in Fig. 1. Compared with the control group, reductions of 22.3%, 21.2%, and 31.5% in sperm number were observed in the TL plus 0.4 Gy, 0.8 Gy, and 1.0 Gy groups, respectively. The induction of sperm head abnormalities is a well-established sensitive assay for assessing reproductive tissue response to ionizing radiation [23]. In our study, a significant enhancement in the number of abnormal spermatozoa was observed in the TL group and TL plus irradiated groups ($P<0.01$) compared to the control group, but there was no radiation dose-dependent response, indicating that sperm malformation could be mainly attributed to the effect of microgravity.

[Figure 1: see original paper] Effects of microgravity on sperm count and sperm abnormality induced by carbon-ion irradiation. Mice were treated with tail suspension and whole-body exposure to carbon ions, 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 Changes Induced by Carbon-Ion Irradiation

To evaluate alterations in spermatogenic function following exposure to ionizing radiation and simulated microgravity, the numbers of spermatogonia or primary spermatocytes per seminiferous tubule in treatment groups are quantitatively shown as ratios of the control group in Fig. 2A. The average numbers of spermatogonia in groups 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, spermatogonia in the 1.0 Gy irradiation plus TL-treatment group decreased by 39.4% compared to the control group. Additionally, the numbers of primary spermatocytes were

remarkably reduced in the combined treatment groups receiving over 0.4 Gy irradiation (P<0.05). The photomicrographs in Fig. 2B illustrate abnormal globular (multinucleated) cells in the seminiferous tubules of animals administered simulated microgravity combined with carbon-ion irradiation at doses over 0.4 Gy.

[Figure 2: see original paper] 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, then sacrificed 24 h 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 seminiferous tubules when animals were administered simulated microgravity combined with carbon-ion irradiation; black block arrows indicate globular (multinucleated) cells.

3.3 Effects of Microgravity on Expression of Apoptotic Bcl-2 Family Proteins 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 includes anti-apoptotic members such as Bcl-xL and pro-apoptotic members such as Bax. The balance of the Bax/Bcl-xL ratio determines whether germ cells undergo death or survival following simulated microgravity and radiation exposure. Fig. 3A shows that over 0.8 Gy of irradiation plus microgravity induced a notable decrease in Bcl-xL protein levels compared to the control group. There were approximately 1.58- and 1.62-fold increases in the relative Bax/Bcl-xL expression ratio in the 0.8 Gy and 1.0 Gy irradiation plus TL-treatment groups compared with the control group.

[Figure 3: see original paper] 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 Caspase-3 Expression Induced by Carbon-Ion Irradiation

Caspase-3 acts as a major executor of apoptosis. As shown 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), and 1.0 Gy (P<0.01) plus TL-treatment compared to the control group.

[Figure 4: see original paper] 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 significant 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 tail suspension and subsequent carbon-ion irradiation, numbers of spermatozoa and spermatogonia were significantly reduced according to H&E staining in groups receiving over 0.4 Gy irradiation plus microgravity compared to the control group. This finding demonstrated that the spermatogenesis process can potentially be blocked. However, there were no statistically significant differences in the TL-treated mice alone or in lower dose irradiation combined with TL-treated mice. We propose that short-duration tail or hind-limb suspension may alter serum hormonal levels such as testosterone (data not shown), FSH, and LH in Leydig cells [11], as well as increase sperm abnormality (Fig. 1), but may fail to cause a notable decrease in spermatogenetic cells including spermatogonia or primary spermatocytes. Hence, the deleterious effects on spermatogenesis in our study may be attributed primarily 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 ions may exhibit chronic or delayed damage [19].

Furthermore, it is noteworthy that sperm count was partially but not significantly decreased in the TL and lower dose irradiation treatment groups, indicating that modeled microgravity did not markedly interfere with the increased damage to seminiferous tubules induced by carbon-ion irradiation. The results from H&E staining showed that globular (multinucleated) cells occurred in over 0.4 Gy irradiation groups with differing distributions in the spermatogenic epithelium. Multinuclear germ cells 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 antiorthostatic 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 proper differentiation, and individualize gametes [28]. Our data previously indicated that carbon-ion irradiation induced a dose-dependent increase in the apoptotic index under simulated microgravity conditions, and that apoptotic cell values in the 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 pro-apoptotic versus anti-apoptotic 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 the 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 in 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, limited information is available concerning the relationship between high-LET radiation and microgravity. Further investigation of the interactions between simulated microgravity and irradiation 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.

References

1. Kierfer J, Proess H D. Mutat Res, 1999, 430: 299–305.
2. Smith S M, Wastney M E, O'Brien K O, et al. FEBS Lett, 2000, 477: 135–140.
3. Fitts R H, Riley D R, Widrick J J. J Appl Physiol, 2000, 89: 823–839.
4. Sonnenfeld G. Curr Pharm Biotechnol, 2005, 6: 343–349.
5. Tedeschi G, Pagliato L, Negroni M, et al. Cell Biol Int, 2003, 31: 1605–1610.
6. Masini M A, Prato P, Scarabeli L, et al. Adv Space Res, 2011, 47: 575–581.
7. Hadley J A, Hall J C, O'Brien A, et al. J Appl Physiol, 1992, 72: 748–759.
8. Sekine E, Okada M, Matsufuji N, et al. Mutat Res, 2008, 652: 95–101.
9. Kamiya et al. [citation incomplete in original]
10. Zhao W P, Zhang H, Wang Y L, et al. J Radiat Res Radiat Process, 2008, 26: 343–347.
11. Zhang H, Zheng R L, Wang R Y, et al. Mutat Res, 1998, 398: 27–31.
12. Rao D V, Narra V R, Howell R W, et al. Radiat Res, 1991, 125: 89–97.
13. D'Souza U J. Asian J Androl, 2003, 5: 217–220.

14. Silaev A A, Kasparov A, Korolev V V, et al. Morphol Pathomorphol, 1977, 46: 27–32.
15. Russell W L, Bangham J W, Russell L B. Genetics, 1998, 148: 1567–1578.
16. Liu G, Gong P, Bernstein L R, et al. Crit Rev Toxicol, 2011, 35: 249–258.
17. [Reference 17 missing in original]
18. [Reference 18 missing in original]
19. [Reference 19 missing in original]
20. [Reference 20 missing in original]
21. [Reference 21 missing in original]
22. Chen et al. [citation incomplete in original]
23. [Reference 23 missing in original]
24. [Reference 24 missing in original]
25. [Reference 25 missing in original]
26. Forsman et al. [citation incomplete in original]
27. [Reference 27 missing in original]
28. [Reference 28 missing in original]
29. [Reference 29 missing in original]
30. Zou et al. [citation incomplete in original]

Acknowledgments

We thank the accelerator crew at the HIRFL, National Laboratory of Heavy Ion Accelerator in Lanzhou.

Note: Figure translations are in progress. See original paper for figures.