Letter to the Editor

Carbon Ion Irradiation Induces Reduction of β-tubulin in Sperm of Pubertal Mice*

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Microtubules are involved in a variety of cellular functions such as cell division, intracellular transport, maintenance of cell polarity and flagella and ciliary motility. The heterogeneity of tubulin and microtubule-associated proteins is responsible for these different microtubule functions. Many studies have confirmed that the structure and function of the different α-tubulin and β-tubulin subunits can affect the microtubule. The sperm axoneme microtubule has linear fiber filaments which are polymerized by heterodimeric α and β-tubulin, each with a molecular mass of approximately 50 kD[1].

The cause of infertility due to ion radiation may be related to low sperm motility, because exposure to ion radiation can impair sperm swimming behavior, and ion radiation has been shown to reduce the total number of sperm and the number of motile sperm in humans[2]. Male infertility is mainly resulted from low sperm motility, however, the relationship between the pathological state of sperm induced by radiation and tubulin changes has not been reported. It has been reported that tubulin affects the morphological state of the sperm tail in human sperm pathology. This study suggested a relationship between low sperm motility after heavy ion radiation (HIR) and a reduction in β-tubulin expression in pubertal mice. Such a relationship may be used to probe the effect of HIR on reproductive health. The results of this study are expected to aid studies on radiation protection in future space environment radiation and HIR cancer therapy.

A total of 40 infant male Swiss Webster mice (Lanzhou University School of Medicine, China) about weighing 12-14 g (21 d old) were used. All animals were kept in 22±2 °C, 60%±10% humidity and light: dark cycle 12 h: 12 h. All feeding procedures were approved by Lanzhou University School of Medicine. Mice were randomly divided into four groups including control (0 Gy), 0.5, 1, and 2 Gy groups. The mouse was fixed in a chamber and its whole-body was irradiated with carbon ion beam at 200 MeV/U and 31.3 keV/μm of the beam entrance, with a dose rate approximately 0.5 Gy/min at the Heavy Ion Research Facility in Lanzhou (HIRFL, Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou, China).

Ten mice from each group were used at 35 days after irradiation. Mice were killed by cervical dislocation. The epididymides of each mouse were taken out. For sperm sampling, the cauda epididymidis was in a pre-warmed petri dish containing 5 mL saline at 37 °C for 10 min to allow the sperm to swim up. The sperm suspension (1 mL) was transferred into 2 mL microcentrifuge tubes.

Sperm motility was measured as the ratio between the upper and lower suspension. Inactive sperm cells were considered to be nonmotile, while active sperm cells were considered to be motile. The percentage of sperm motility was calculated using the number of motile sperm cells over the total number of sperm cells (both motile and nonmotile). Sperm viability was evaluated by acridine orange fluorescence of sperm nuclei. Caudal sperm were smeared on clean glass side. The smear was stained with 4 ng/mL acridine orange (Sigma, St. Louis, MO, USA) for 2 min followed by counterstaining with 0.5 ng/mL propidium iodide (Sigma, St. Louis, MO, USA) for 1 min. The stained slides were examined in dark room using a fluorescence microscope (Nikon, 80i, Japan). Live sperm fluoresced to green in the head and midpiece region, while damaged sperm fluoresced to red in the head region[3]. The sperm

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smears soaked in 3% H_2O_2 and incubated for 30 min; 1% Triton X-100 for 15 min; 5% BSA blocked for 25 min at room temperature after the dropwise addition, rabbit polyclonal IgG anti-β-tubulin (1:500) 4 °C overnight; dropwise addition, donkey anti-rabbit IgG (1:100) antibody Alexa Fluor 555 (cat. # A-21432) (Invitrogen life technologies, California, USA), 37 °C incubated 1 h; the above steps with a 0.01 mol/L (pH 7.2-7.4) PBS washed 2 times, 5 min/time; DAPI (5 μg/mL, Sigma, St. Louis, MO, USA) staining 5 min and PBS washed 2 times, 5 min/time, a glycerin- sodium bicarbonate wet sealant Fengpian. Confocal laser microscope (LSM, Zeiss, Germany) and camera sperm on a Now red fluorescence was positive[4].

The data were analyzed with SPSS statistical software (SPSS 19.0, Inc., Chicago, IL).

The infancy period of mice is from birth to five weeks of age, the puberty period is from 5 to 7 weeks of age and the period of adulthood is from 8 to 30 weeks of age. Infancy to adulthood is a critical period of growth and development in mice, as this is a stage when body size and organ weight develop quickly, and sperm motility is higher than at any other stage. The period of spermatogenesis in mice takes 35 d[5], and mature sperm begin to increase morphologically in the cauda epididymis at approximately 5.5 weeks of age. Five weeks after irradiation were chosen to investigate the chronic effects of ion radiation on the caudal sperm of infant mice, especially during puberty when sexual maturation takes place. To investigate the long-term effects of HIR on caudal sperm in pubertal mice, we examined sperm viability and motility. Significant reductions in sperm motility (Table 1) were observed in the groups exposed to irradiation (P<0.001). There was a marked decrease in sperm viability in the irradiated groups (0.5 Gy, P<0.01; >0.5 Gy, P<0.001; Table 1). Figure 1 shows AO/PI staining in live sperm. These results suggest that HIR can affect spermatogenesis in infant mice, by reducing sperm viability and motility at 35 d after irradiation. Sperm viability significantly decreased, demonstrating a higher dose of carbon ion that notably reduced the percentage of live sperm at 35 d after radiation. Sperm motility is one of the major determinants of male fertility and it can be used to predict the penetration ability of sperm[6]. In the present study, sperm motility data showed a significant decrease with increasing dose, which is consistent with findings of previous studies. Bartoov et al. reported the reduction in the sperm motility of radiation-exposed workers. Ultra-morphologic defects were evident on the sperm nucleus and fertility potential was adversely affected among the exposed workers[7]. Au et al. found that various sperm motion parameters decreased with increasing dose and *Euscepes postfasciatus* sperm motility after gamma radiation[8].

We investigated the expression of β-tubulin in relation to the possible position and distribution of sperm using immunofluorescence. When sperm were labeled with an immunofluorescent dye to detect β-tubulin protein expression, β-tubulin protein expression was clearly visible in the sperm midpiece and tail in control sperm. A little β-tubulin protein expression was detected in the irradiated groups, and was undetectable by microscopy in the 2 Gy group (Figure 2). The absence of detection of β-tubulin protein in Figure 2D was possibly caused by the lower sensitivity of immunofluorescence staining as compared with immunoblotting, however, the trend for differences between the control and irradiated sperm was consistent. The epididymide index can reflect epididymide development. Recently, many studies have confirmed that tubulin expression is related to sperm motility[9]. The motility of mammalian sperm relies on movement of the sperm flagellum, a whip-like appendage with a central cytoskeletal structure called the axoneme, and its function requires precise microtubule organization. β-tubulin plays an important role in the process of spermatogenesis and maintenance of sperm motility. Huang et al. reported significantly higher expression of β-tubulin in the testes of mature boars which may indicate that β-tubulin is essential for sperm formation in porcine testes[10]. Popodi et al. described sea urchin sperm flagellar tubulin isoforms which have been directly implicated in motility and may

Table 1. Effects of Whole Body Carbon Ion Irradiation on Caudal Sperm Viability and Sperm Motility of the Pubertal Mice at 35 d after Irradiation

Items	Control	0.5 Gy	1 Gy	2 Gy
Viability	0.85±0.02	0.76±0.02*	0.70±0.02**	0.68±0.01**
Motility	0.69±0.03	0.38±0.03**	0.31±0.02**	0.25±0.03**

Note. Values represent the average±SE. from ten mice per group. $^{*}P$<0.01, $^{**}P$<0.001 as compared with control group.

play a more structured role in motility[9]. β-tubulins utilized in motile '9+2' axonemes contain a C-terminal sequence 'axoneme motif' which can support the production of functional motile sperm[1].

The aim of this study was to investigate the effects of HIR on sperm motility and assess the status of sperm with absent or severely reduced motility in relation to β-tubulin. The differential effects on sperm motility and viability suggest that HIR has long-term effects on sperm formation and development and possible effects on the epididymis, where sperm motility develops. Typical distribution of the β-tubulin protein in normal sperm is shown in Figure 2A. Regular β-tubulin signals were observed and β-tubulin protein was distributed in the sperm midpiece and tail. This was positively correlated with weakened sperm motility due to increasing radiation dose, indicating that the reduction in β-tubulin expression may lead to reduced sperm motility.

In conclusion, the present study has proven, for the first time, that exposure to HIR harms caudal sperm in pubertal mice at 35 d after irradiation.

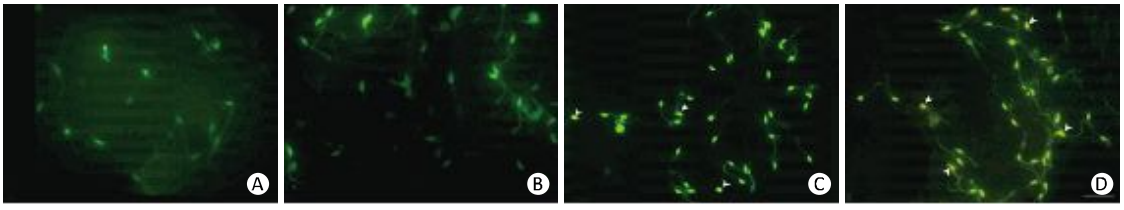

Figure 1. Photomicrographs of live and dead sperm. Live sperm fluoresced to green in the head and midpiece region, while damaged sperm fluoresced to red in the head region (arrows). Control (A), 0.5 Gy (B), 1 Gy (C), 2 Gy (D). AO/PI staining was performed at 40× magnification, scale bar=10 μm.

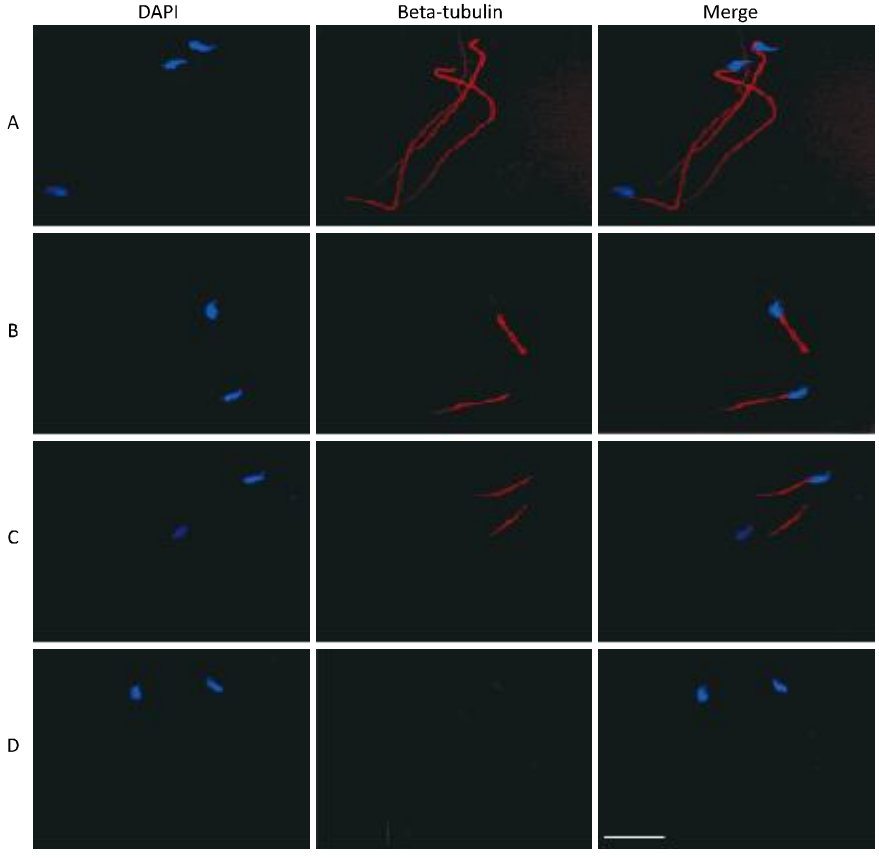

Figure 2. Immunofluorescence staining of β-tubulin protein in the sperm of different experimental groups. Control (A), 0.5 Gy (B), 1 Gy (C), 2 Gy (D). The staining was performed at 630× magnification, scale bar=20 μm. The DNA is detected in the sperm nucleus (blue), the midpeice and tail are localized with β-tubulin (red).

Furthermore, the results of this study reveal that sperm motility is highly vulnerable to HIR and absent or weak β-tubulin labeling seems to be associated with absent or weak sperm motility.

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