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Differential proteome and gene expression reveal response to carbon ion irradiation in pubertal mice testes

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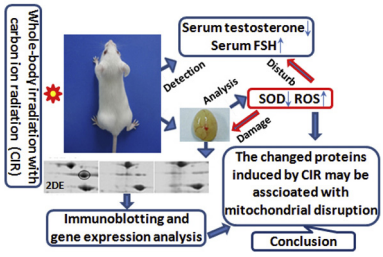
HIGHLIGHTS

- Proteomics of pubertal mice testes after carbon ion radiation were examined.
- Differential proteins in 2-DE gels were investigated 14 days after irradiation.
- Eight proteins were identified among the differentially expressed protein spots.
- The relationship between mRNA and the abundance of proteins were confirmed.
- These proteins may lead to new insights into the information of CIR toxicity.

GRAPHICAL ABSTRACT



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ABSTRACT

Heavy ion radiation, a high linear energy transfer (LET) radiation, has been shown to have adverse effects on reproduction in male mice. The aim of this study was to profile and investigate the differentially expressed proteins in pubertal male mice testes following carbon ion radiation (CIR). Male mice underwent whole-body irradiation with CIR (1 and 4 Gy), and MALDI-TOF/TOF analysis was used to investigate the alteration in protein expression in 2-DE (two-dimensional gel electrophoresis) gels of testes caused by irradiation after 14 days. 8 differentially expressed proteins were identified and these proteins were mainly involved in energy supply, the endoplasmic reticulum, cell proliferation, cell cycle, antioxidant capacity and mitochondrial respiration, which play important roles in the inhibition of testicular function in response to CIR. Furthermore, we confirmed the relationship between transcription of mRNA and the abundance of proteins. Our results indicated that these proteins may lead to new insights into the molecular mechanism of CIR toxicity, and suggested that the gene expression response to CIR involves diverse regulatory mechanisms from transcription of mRNA to the formation of functional proteins.

Abbreviations: HIR, heavy ions radiation; 2-DE, two-dimensional gel electrophoresis; MALDI-TOF-TOF, matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry; HIRFL, heavy ion research facility in Lanzhou; CCB, colloidal coomassie blue G 250; TBS, tris buffer saline; DAB, diaminobezidine; HRP, horseradish peroxidase; FSH, follicle-stimulating hormone; LH, luteinizing hormone; GRP78, 78 kDa glucose-regulated protein; VCP, valosin-containing protein, isoform CRA_a; ACO, aconitate hydratase–mitochondrial precursor; PKM1/M2, pyruvate kinase isozymes M1/M2 isoform 1; GSTA3, glutathione-*S*-transferase A3; GSTP1, glutathione *S*-transferase P 1; SOD1, Cu/Zn superoxide dismutase.

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1. Introduction

The biological effects of high linear energy transfer (LET) heavy-ion radiation are more pronounced than low-LET radiation, such as X-rays or γ-rays (Duan et al., 2008). Heavy ions have greater potential for application in various disciplines, especially in heavy ion radiotherapy which involves trends in tumor localization radiotherapy and its related biological research, and has become a hot topic in research related to radiobiology and radiation medicine (Tsujii and Kamada, 2012; Ohno, 2013). However, the negative effects of heavy ion therapy cannot be ignored, as studies have shown that heavy ions have a stronger cell killing effect than X- and γ-rays (Sekine et al., 2011; Varès et al., 2011). In addition, ionizing radiation can also cause damage to the body's tissues and organs, and can generate reactive oxygen free radicals, resulting in bond breakage in biological molecules, the role of bioorganic molecules, enzyme inactivation, DNA damage, changes in biofilm permeability and even death (Datta et al., 2012).

Spermatogenesis is a complex process of male germ cell proliferation and maturation from spermatogonia to spermatozoa (Santos and Kim, 2010). However, irradiation can influence spermatogenesis, as doses as low as 0.1 Gy are known to cause damage to spermatogenia (Gong et al., 2013). Less than 2% of men who have received total-body irradiation are able to father a child later in life (Otala et al., 2004). Pediatric and adolescent patients with tumors treated with radiotherapy may have deficiencies in the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), as well as testosterone (Zhang et al., 2006). Therefore, with the advent of new radiotherapy modalities other than heavy ions, there has been a considerable improvement in the survival rate of cancer patients, and men of child-bearing age undergoing radiotherapy are often concerned about the possibility of future children. Thus, it is important to ascertain whether high LET radiation exposure induced spermatogenesis dysfunction, and pay more attention to the reproductive potential and the possible genetic alteration in the spermatogenic cells of these patients.

The analysis of male and female reproductive functions has gained impetus deepening our insight into the normal and pathological complexities of infertility and its causes; however, much still remains to be elucidated. To understand these biological complexities, the physiological function of each protein at the tissue level needs to be carefully addressed. Remarkable advances in proteomics technology, especially in the protein/peptide resolution technique and mass spectrometry have offered unparalleled opportunities for global and targeted analyses of the expression, regulation and modification of proteins in various biological systems (Kolialexi et al., 2008). In recent years, the reference proteomes of male reproductive cells/tissues/organs such as sperm (Gilany et al., 2011), epididymis (Li et al., 2010) and testis (Li et al., 2011) have been obtained and these have enriched our knowledge on the proteins expressed in these cells/tissues/organs. Efforts are also being made to identify the proteins which contribute to the pathology of various reproductive health disorders such as azoospermia and oligozoospermia in men (Upadhyay et al., 2012).

In this study, we used a proteomic approach based on a two-dimensional electrophoresis (2-DE) reference map to determine alterations in protein expression in the testes of pubertal Swiss-Webster mice subjected to CIR. In order to further confirm the proteomic results, the gene expression changes were analyzed. Serum testosterone and serum FSH levels were measured to evaluate the toxic effects of CIR on pubertal mice testes. Superoxide dismutase (SOD) and intracellular ROS were measured to confirm mitochondrial disruption and oxidative stress induced by CIR in pubertal mice testes. ATP levels were examined to estimate testis energy balance in pubertal mice testes following CIR. Integration of the proteome, gene analysis and biochemical data provided evidence to help understand the underlying mechanisms of CIR toxicity in male pubertal patients following CIR.

2. Materials and methods

2.1. Animals

Male mice about weighing 18–22 g (4 weeks) of Swiss-Webster mice provided by Lanzhou Medical College (Lanzhou, China) were used under identical breeding conditions, all animals were kept in 22 ± 2 °C, 60 ± 10% humidity and light: dark cycle 12 h:12 h. A total of 30 mice were randomly divided into three groups including control (0 Gy), low dose group (1 Gy) and high dose group (4 Gy), each group constituted 10 mice. The studies were approved by the Institutional Animal Care Committee.

2.2. Irradiation procedure and animals treatment

Mouse was fixed in a chamber and whole-body irradiated with carbon ion beam at 300 MeV/U and 31.3 keV/μm of the beam entrance, with dose rate was approximately 0.5 Gy/min at 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. Ten mice from each group were weighed and killed by cervical dislocation at 14 days after irradiation. Blood was collected and centrifuged at 2000*g* at 4 °C for 15 min. Serum was stored at −80 °C until analysis. The testes were weighed and frozen immediately in liquid nitrogen and stored at −80 °C until analysis.

2.3. Serum testosterone and serum FSH levels

Concentrations of serum testosterone and serum FSH were measured using ELISA kits (Elabscience Co., Wuhan, China).

2.4. Histopathologic quantification and evalution of testicular cells apoptosis

The testes were fixed in 4% paraformaldehyde solution (4 g paraformaldehyde in 100 mL PBS) and then embedded in paraffin blocks. Testicular sections of 5 μm were cut, stained with hematoxylin–eosin (H&E) (Heechul et al., 2008) and observed under a light microscope (Zeiss, Germany). A score of 0–10 was used for each tubule according to the number of damaged cells in the seminiferous tubules as described by Trivedi et al. (2010). Histological quantification of spermatogenesis in the testicular sections was performed by assigning a Johnsen score (Trivedi et al., 2010; Li et al., 2013a).

The terminal dUTP nick end-labeling (TUNEL) assay was used to evaluate testicular cells apoptosis. Testicular sections were deparaffinized, rehydrated, and then incubated with 20 mg/mL proteinase K for 20 min and rinsed in Tris buffer saline (TBS). The steps for TUNEL staining were carried out using the In Situ Cell Death Detection, POD Kit (Roche, Mannheim, Germany). The sections were incubated with the diluted TUNEL reaction mixture (TdT-enzyme was diluted 1:10 with sterile water) for 60 min at 37 °C. The sections were blocked with 3% BSA for 25 min at room temperature, incubated with the secondary antifluorescein-POD-conjugate for 30 min, and then labeled with diaminobenzidine (DAB). After each step, the sections were thoroughly washed with TBS. The sections were then counterstained with hematoxylin, dehydrated, cleaned and mounted. Positive cells were stained brown. Quantitative analysis of testicular cell apoptosis was estimated according to Lysiak et al. (2000). Apoptotic cells were TUNEL-positive and the positively stained nuclei in 30 circular seminiferous tubule cross-sections per testis section were counted.

2.5. Protein preparation, two-dimensional gel electrophoresis (2-DE) and image analysis

Frozen testis tissues were prepared for extraction of proteins and mRNA. The testicular tissue was treated with a lysis buffer containing 7 M urea, 2 M thiourea, 4% (W/V) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (Chaps), 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 with bovine serum albumin (Sigma) as a standard.

Protein samples (600 μg) from three groups were dissolved in 350 μL IEF sample buffer [8 M urea, 2% CHAPS, 65 mM DTT, 0.2% (w/v) Bio-Lyte 3-10 ampholytes, and Bromophenol Blue (trace)] and loaded onto an IPG strip (Immobiline 17 cm DryStrip pH 3–10, Bio-Rad, USA), then covered with 1 mL of mineral oil for IEF using a Protean IEF cell (Bio-Rad Laboratories, USA). Procedure for IEF was accordied to the following program: 14 h at 50 V; 250 V for 1 h; 1000 V for 1 h; 9000 V for 6 h; 9000 V for 80,000 V h. After being focused, the IPG strips were equilibrated for 15 min in equilibration solution [6 M Urea, 30% (v/v) glycerol, 2% (w/v) SDS, 50 mM Tris–HCl, pH 8.8, 1% (w/v) DTT] and then alkylated for a further 15 min in this solution without DTT but with 2.5% iodoacetamide. Electrophoresis of reduced and alkylated samples was carried out on Protean II xi Cell (Bio-Rad Laboratories, USA) using 12% polyacrylamide SDS-PAGE gels [30% acrylamide solution, 1.5 M Tris (pH 8.8), 10% SDS, 10% ammonium persulfate, TEMED and ultrapure water]. The second dimension electrophoresis, SDS-PAGE, was carried out to following program: at 25 mA/gel until bromophenol blue eluted from the gel at a room temperature with water cooling (Li