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Proteome analysis for profiling infertility markers in male mouse sperm after carbon ion radiation

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ABSTRACT

Ion radiation or radiotherapy is used to treat male patients with oligozoospermia, azoospermia, temporarily infertility, or even permanent infertility. The present study aims to investigate the potential infertility mechanism of sperm in mice after carbon ion radiation (CIR). The caudal epididymal sperm of male mice whole-body irradiated with carbon ion beam (0.5 Gy and 4 Gy) were used 7 days after irradiation. A two-dimensional gel electrophoresis approach was employed to investigate the changes in protein expression in the caudal edididymal sperm. Spot detection and matching were performed using the PDQuest 8.0 software. The criteria used to select spots for the analysis were more than a threefold difference in protein quantities (normalized spot volume), which allowed the detection of six differentially expressed proteins. Protein identification was performed using MALDI-TOF-TOF. Six specific proteins were identified by searching the NCBI protein sequence database. Among these proteins, HSP 70-2, PLC, GPX4, β-tubulin, and GAPDHS were associated with sperm motility, which can affect fertility. β-tubulin is important in axoneme migration flagellar movement and regulation, and GAPDHS is related to sperm energy supply. We analyzed their expressions using immunoblotting and immunofluorescence. The changes in sperm protein expression after CIR are mainly associated with motility. These proteins are potential markers for the mechanisms of infertility in space or radiotherapy.
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1. Introduction

The biological effects of radiation can damage the DNA, proteins, and lipids in cells (Zhang et al., 2006). Although heavy ion accounts for only 1% of the space environment (Blakely and Kronenberg, 1998), the biological effects of heavy ion beams, such as carbon, cause more damage (Lee et al., 2005; Pathak et al., 2007) and show a stronger ability to inactivate cells than low linear energy transfer radiation such as X- or γ-rays (Blakely and Kronenberg, 1998; Murakami et al., 2001).

Humans are exposed to ionizing radiation (IR) under various circumstances, such as cosmic radiation, diagnostic X-rays, and radiotherapy for cancer. IR impairs spermatogenesis and causes mutations in germ cells (Xu et al., 2008). Although radiotherapy is effective for treating cancer, patients often complain of azoospermia or infertility. Short-term and long-term effects of radiation on the number and structure of epididymal spermatozoa were also reported (Songthaveesin et al., 2004). The rise of heavy ion radiotherapy technology in recent years has provided a new therapeutic approach for cancer patients; however, this technology needs further assessment because it damages normal tissues (Krämer and Scholz, 2000). Recent studies have focused on the changes in the male reproduction system after irradiation. Several studies demonstrated that radiotherapy of spermatogonial stem cells for malignancy management causes male infertility (Wang et al., 2005). Radiation-induced chromosomal aberrations of spermatogonia and spermatocytes can be transmitted into the spermatozoa, causing asthenospermia, hypospermia, and teratospermia (Zhang et al., 2008). These studies are focused at the morphological level (e.g., motility, morphology, concentration, and DNA fragmentation). However, intrinsic molecular level studies are rare. To date, little is known about the detailed clinical nature of sperm dysfunction in “hidden” male factors of infertility after irradiation.

Two-dimensional gel electrophoresis (2-DE) provides a technical basis for sperm proteomics research. Based on the isoelectric point and molecular weight separation of proteins, this technique provides a visual reference for the assessment of the sperm proteome by displaying up to 2000 protein spots in the 2-D gel

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(Naaby-Hansen et al., 2001; Martínez-Heredia et al., 2006). Proteome analysis could be used to determine the changes in sperm protein expression after irradiation, explore the mechanism of "hidden" male factors of infertility, and identify infertility-related protein markers in sperm after irradiation.

The present study applied a proteomic approach to investigate the protein composition and to establish a 2-D PAGE reference map for caudal epididymal sperm proteins in mice after carbon ion radiation (CIR). A 2-DE approach was employed to examine the changes in protein expression in the epididymal sperm of Swiss Webster mice after CIR. We constructed a 2-DE map of whole proteins in mouse caudal sperm. We investigated the proteomic changes in mouse caudal sperm after CIR and determined the relationship between the proteomic changes and irradiation doses. We also analyzed the function of differential proteins after CIR and the expressions of β-tubulin and GAPDHS proteins.

2. Materials and methods

2.1. Animals

A total of 36 adult male Swiss webster mice (Lanzhou University School of Medicine, China) weighing 30–35 g were used. All animals were kept in 22 ± 2 °C, 60 ± 10% humidity and light:dark cycle 12 h:2 h. All fed procedures were approved Lanzhou University School of Medicine. Mice were randomly divided into three groups including control (0 Gy), 0.5, 4 Gy group, each group constituted twelve mice.

2.2. Irradiation procedure

Mouse was fixed in a chamber and whole-body irradiated with carbon ion beam at 200 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 (Li et al., 2007). Data were controlled automatically by a microcomputer during irradiation. Particle fluence was determined from an air-ionization chamber signal according to the calibration of the detector (PTW-UNIDOS, PTW-Freiburg Co., Wiesbaden, Germany).

2.3. Sperm counts, sperm motility and sperm viability analysis

Twelve mice from each group were used 7 days after irradiation. Mice were killed by cervical dislocation. The epididymides of each mouse were taken out. The fat and connective tissues adhering to epididymides were removed. For sperm sampling, the right epididymidis was placed in PBS, the epididymidis separated from the tissue with a surgical blade. The epididymidis was cut into small pieces, and cultured in 3 mL DMEM supplemented with 10% fetal bovine serum for 30 min at 37 °C to allow the sperm to swim up (Li et al., 2010). The sperm suspension (1 mL) was transferred into 2 mL microcentrifuge tubes. Sperm counts were used a haemocytometer under light microscope at 200× (Nikon, Japan). The sperm suspension (1 mL) was transferred into 2 mL microcentrifuge tubes. A volume of 100 μL was mixed with 500 μL of the same medium and incubated for 15 min at 37 °C at a microcentrifuge tube. The upper suspension (200 μL) and the lower suspension were then separately collected, and the sperm count was assessed. Sperm motility was measured as the ratio between the upper and lower suspension. Inactive sperm cells at all were considered to be nonmotile, 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) (Yan et al., 2007). Sperm viability was tested using MTT assay. Sperm suspension (50 μL) was mixed with 25 μL MTT reagent (5 mg/ml in PBS) and incubated in 96 microplate well at 37 °C for 2 h. After centrifugation and pipeting exhaustively, then added 100 μL DMSO to dissolve formazan. Sperm activity was estimated as an absorbance at 490 nm (Tecan M200, Switzerland) (Ohatni et al., 2004).

2.4. Intracellular reactive oxygen species

Sperm suspension (50 μL) loaded with 10 μM DHE (dihydroethidium) and 5 μM 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Sigma Chemical, St. Louis, MO, USA), respectively. After incubation for 30 min in the dark, then, sperms were detached, and resuspended in 1 ml PBS. Cellular ROS in 10,000 cells as a result of the oxidation of DCFH-DA was measured (excitation, 470 nm; emission, 530 nm, Thermo Varioskan Flash 3001, USA) (Matás et al., 2010). Cellular ROS in 10,000 cells as a result of the oxidation of DHE was measured (excitation, 488 nm; emission, 595 nm, Thermo Varioskan Flash 3001, USA) (Vanden Hoek et al., 1998).

2.5. Extracted proteins and measured protein concentration

Sperm was treated with a lysis buffer containing 7 mol/L urea, 2 mol/L thiourea, 4% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (Chaps), 2% (w/v) dithiothreitol (DTT) in the presence of 1% (w/v) 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.

2.6. Two-dimensional gel electrophoresis (2-DE) and image analysis

300 μg protein samples and loading buffer [8 M urea, 2% CHAPS, 65 mM DTT, 0.2% (w/v) Bio-Lyte 3-10 ampholytes, and Bromophenol Blue (trace)] were mixed up to a total volume 350 μL. The mixtures were transferred to the tray and put in 17 cm IPG strip, then covered with 1 mL of mineral oil for IEF using a protean IEF cell (Bio-Rad Laboratories, USA). Procedure for IEF was according 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 Vh. After focusing, 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 equilibration solution [6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 50 mM Tris–HCl, pH 8.8, 2.5% (w/v) iodoacetamide]. Lectrophoresis of reduced and alkylated samples was carried out on Protean II xi Cell (BioRad, 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 running out of the gel at a room temperature with water cooling (Jia et al., 2007; Hooven and Baird, 2008). After electrophoresis, the gels were stained with colloidal coomassie blue G 250 (CCB) procedure for at least 14 h. Following staining, the gel was neutralized with 0.1 M Tris/phosphoric acid (pH 6.5) for 1–3 min, then destained with 25% methanol. Three 2-DE gels were prepared for each sample and were then scanned with UMAX scanner, and those data were saved as a TIFF image file for further image analysis.

Spot detection and matching were performed using the PDQuest 8.0 software (Bio-Rad Laboratories, USA). The spots detected automatically by the software were visually inspected. Spot filtering and editing were performed manually to remove artifacts and to correct for spots that did not split correctly. The gel replicates were subsequently combined into average gels, which represented spots that were reproducibly present on all of the replicate gels. The criteria used to select spots for the analysis were more than a threefold difference in protein quantities (normalized spot volume) between control and irradiation groups. ANOVA (version 19.0; SPSS Inc., Chicago, IL, USA) was used to test the significance of the normalized volume in total density of identified proteins in all gels.

2.7. In-gel trypsin digestion of proteins and MALDI-TOF/TOF MS analysis

Spots were manually cut from 2-DE gels, destained for 20 min in 30 mM potassium ferricyanide/100 mM sodium thiosulfate (1:1 (v/v)) and washed with Milli-Q water until the gels were destained. The spots were incubated in 0.2 M NH_4HCO_3 for 20 min and then lyophilized. Each spot was digested overnight in 12.5 ng/ml trypsin in 25 mM NH_4HCO_3. The peptides were extracted three times with 60% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA). The extracts were pooled and dried completely by a vacuum centrifuge.

MS and MS/MS data for protein identification were obtained by using a MALDI-TOF-TOF instrument (4800 proteomics analyzer; Applied Biosystems, Foster City, USA). Instrument parameters were set using the 4000 Series Explorer software (Applied Biosystems, Foster City, USA). The MS spectra were recorded in reflector mode in a mass range from 800 to 4000 with a focus mass of 2000. MS was used a CalMix5 standard to calibrate the instrument (ABI 4700 Calibration Mixture, Applied Biosystems, Foster City, USA). For one main MS spectrum 25 subspectra with 125 shots per subspectrum were accumulated using a random search pattern. For MS calibration, autolysis peaks of trypsin ([M+H]+842.5100 and 2211.1046) were used as internal calibrates, and up to 10 of the most intense ion signals were selected as precursors for MS/MS acquisition, excluding the trypsin autolysis peaks and the matrix ion signals. In MS/MS positive ion mode, for one main MS spectrum, 50 subspectra with 50 shots per subspectrum were accumulated using a random search pattern. Collision energy was 2 kV, collision gas was air, and default calibration was set by using the Glu1-Fibrino-peptide B ([M+H]+ 1570.6696) spotted onto Cal 7 positions of the MALDI target. Combined peptide mass fingerprinting PMF and MS/MS queries were performed by using the MASCOT search engine 2.2 (Matrix Science, Ltd, London, UK.) embedded into GPS-Explorer Software 3.6 (Applied Biosystems, Foster City, USA) on the NCBI database with the following parameter settings: 100 ppm mass accuracy, trypsin cleavage one missed cleavage allowed, carbamidomethylation set as fixed modification, oxidation of methionine was allowed as variable modification, and MS/MS fragment tolerance was set to 0.4 Da. A GPS explorer protein confidence index ≥ 95% were used for further manual validation.

2.8. Immunoblotting and immunofluorescence

Protein extracted from the sperm (60 μg/10 μL) was subjected to SDS-PAGE [12% (w/v) polyacrylamide gel] and the resolved proteins were transferred to a PVDF membrane. The membranes were blocked with 5% skimmed milk in Tris

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