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Differential proteome association study of freeze-thaw damage in ram sperm

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ABSTRACT

In this study proteomics analysis was performed to investigate damage caused to ram sperm by the freeze-thaw process. Sperm motility, viability, reactive oxygen species (ROS) and adenosine triphosphate (ATP) content were measured to evaluate sperm quality. Compared with fresh groups, motility, viability and ATP content were all lower in freeze–thawed sperm ($P < 0.001$), and ROS content was higher ($P < 0.001$). Moreover, 25 differential protein spots were detected in two-dimensional gels using PDQuest 8.0 software and the corresponding proteins were identified using matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI-TOF-TOF MS) coupled with searching of the NCBI protein sequence database. Among these proteins, hexokinase1 (HXK1), the enzyme that catalyzes the first step of glycolysis in the sperm glycolytic pathway, is known to be associated with sperm motility. Casein kinase II subunit alpha (CSNK2A2), a serine/threonine-selective protein kinase, is associated with sperm apoptosis. We used immunoblotting and immunofluorescence to analyze the expression and localization of these two proteins. HXK1 and CSNK2A2 expression levels in fresh sperm were significantly higher than that in freeze-thawed sperm ($P < 0.001$). HXK1 and CSNK2A2 were detected in the main part of the sperm flagellum, and the immunofluorescence signal from these proteins was weakened in the freeze-thawed group. Decreased expression of HXK1 and CSNK2A2 may be associated with decreased sperm motility and viability following freeze-thawing.

1. Introduction

Cryopreservation of semen has long been an important tool for preserving male and protecting male fertility in cases such as infertility and malignancy treatments using assisted reproductive technology [27,28]. Although semen cryopreservation has proven to be very valuable, the quality of frozen sperm is highly affected during the process; cryodamage includes reduction in motility [13], cold shock, freezing injury, oxidative stress, alterations in membrane composition, and osmotic stress [24]. These insults to sperm cells include reduced metabolic activities, loss of cytoplasmic proteins, membrane-bound proteins, enzymes and other cellular components, and defects in sperm proteins that may compromise sperm motility, fertilization, and the early events after fertilization [36].

Protein screening methods have demonstrated that the reduction in motility observed in bovine, boar, and human sperm is associated with changes to certain proteins. Lessard et al. [14] reported that the decreased motility of bovine sperm after the freeze–thaw process was associated with loss of P25b, a sperm protein that is associated with the plasma membrane covering the acrosome. Martin et al. [18] found cytochrome c and apoptosis-inducing factor of these two proteins released from the mitochondria in the sub-freezing and thawing process in bovine sperm. Huang et al. [8] and Cao et al. [3] demonstrated that the reduction in motility observed in boar and human sperm following cryopreservation was associated with a decrease in heat shock protein 90 during cooling.

Proteomics is a powerful tool for generating analytical data to

Abbreviations: ROS, Reactive oxygen species; ATP, Adenosine triphosphate; 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, diaminobenzidine; IVF, in vitro fertilization; HXK1, hexokinase 1; CSNK2A2, casein kinase II subunit alpha.

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discover differentially expressed proteins, and elucidating the biochemical and biophysical mechanisms underlying quality control in animals [20]. However, there have been few reports of changes in ram sperm after the freeze–thaw process, especially at the proteomics level. Analyzing changes to sperm proteins at the level of the proteome after freeze–thawing has broad application prospects and research value.

In the present study, sperm motility, viability, reactive oxygen species (ROS), and adenosine triphosphate (ATP) content were measured to evaluate the damaging effects of the freeze-thaw process in ram sperm. Then, two-dimensional polyacrylamide gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI-TOF-TOF MS) were used to analyze the differentially abundant proteins, and to investigate the possible relationship between the differential proteome and damage caused by freeze-thawing of ram sperm. Among the identified proteins, the functions of hexokinase 1 (HXK1) and casein kinase II subunit alpha (CSNK2A2) were further confirmed by immunoblotting and immunofluorescence, because they are associated with sperm motility and apoptosis after thawing. The differential proteins may be useful for identifying related protein markers in ram sperm during the freeze-thaw process, for additional studies on ram sperm proteomics, and for revealing the association between protein expression and cryopreservation damage in ram sperm.

2. Materials and methods

2.1. Semen collection, cryopreservation and thawing

The animal care and sample collection procedures in the present study were approved and conducted according to standards established by the Animal Husbandry and Veterinary Research Institute of Gansu Province (Pingliang, China). Semen from three rams was obtained from five randomly selected mature and healthy Dorset rams (Ovis aries) under the same breeding conditions during a 4-month period. Three ejaculates were obtained from each ram using an artificial vagina with a volume of 0.75–2 mL and the average sperm concentration was 3×10^9 sperm/mL for each ejaculate. The sperm concentration and motility of each pool were subsequently evaluated, using a sperm count plate, by light microscopy (Nikon 80i; Tokyo, Japan) at a 400 × magnification [9]. Only ejaculates with motility >80%, deformation ratio <15%, and sperm concentration $>2.5 \times 10^9$/mL were accepted [23]. To increase semen volume and eliminate variability between samples, ejaculates were pooled, and all experiments were performed with six replicates for each group. Each pool was protected from light, incubated at 37 °C for 30 min, thoroughly mixed, and divided into two fractions. One fraction was used as a fresh sample as a control for protein extraction; the other fraction was diluted at a ratio of 1 part semen to 5 parts extender (at 37 °C), and cryopreserved using the conventional methods of the Animal Husbandry and Veterinary Research Institute of Gansu Province. The main cryopreservation procedure was as follows: The extender (main components: 2.422 g Tris, 1.34 g citric acid, 0.5 g fructose, 500 IU benzylpenicillin, 500 IU streptomycin in 100 mL deionized water) [6,7,29] was added to the semen samples at a 5 × volume. The pH of the extender was adjusted to 7 using Tris. The final sperm concentration in diluted samples was approximately 5×10^8/mL. Diluted samples were slowly cooled from 37 °C to 5 °C in the refrigerator, and allowed to equilibrate for 1 h at 5 °C. During the temperature decline, 20% (v/v) egg yolk and 5.6% (v/v) glycerol were added to the extender when the temperature reached 10 °C and 5 °C, respectively [4]. Next, one fraction of the sample was stored in 0.25 mL straws, which were filled using a semiautomatic filling and sealing machine. Then, the straws were exposed to the steam of liquid nitrogen for 10 min, submerged in liquid nitrogen, and stored in a liquid nitrogen tank for more than 72 h. Immediately before evalution, frozen samples in each straw were thawed in a 37 °C water bath for 8 s, and sperm motility was evaluated [30].

2.2. Sperm motility and determination of ATP concentration

Samples were diluted to 3×10^7 sperm/mL with extender, and sperm count was determined a sperm count plate under a light microscope at 400× (Nikon 80i). Percent sperm motility was calculated as the number of motile sperm over the total number of sperm (both motile and non-motile) [34]. The amount of ATP was measured using an ATP assay kit (Beyotime, Shanghai, China) according to the manufacturer's instructions, and a standard curve of ATP concentration was prepared from a known concentration (1 nM–1 mM) [26].

2.3. Observation of sperm apoptosis

The terminal dUTP nick end-labeling (TUNEL) assay was performed using the *In Situ* Cell Death Detection, POD Kit (Roche, Mannheim, Germany). Briefly, the smears of the final sperm suspension were air-dried on microscope slides at room temperature, and fixed in methanol after the slides had been washed in phosphate buffered saline (PBS) for 10 min three times and treated with 20 mg/mL proteinase K solution for 20 min at 37 °C. Then, the TUNEL reaction was performed in TdT buffer in the presence of dUTP-biotin for 60 min at 37 °C, and then incubated with secondary antifluorescein-POD-conjugate for 30 min. Positive staining was visualized by diaminobenzidine (DAB), and slides were counterstained with hematoxylin.

2.4. Intracellular ROS

The sperm suspension (50 μL) in PBS (1×10^6 sperm/mL) loaded with 5 μM 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Sigma Chemical, St. Louis, USA). After incubation for 30 min in the dark, the sperm was re-suspended in 1 mL PBS. Cellular ROS in 10,000 cells, as a result of DCFH-DA oxidation, was measured using a Varioskan Flash 3001 microplate reader (Thermo, Waltham, Massachusetts, USA) [19] with an excitation of 470 nm and an emission of 530 nm.

2.5. Extraction of sperm proteins

Fresh and freeze-thawed semen samples were centrifuged at 800× g for 10 min at 4 °C. The pellet (consisting of sperm collected by centrifugation of the suspension at 6000× g for 5 min) and supernatants were discarded. Sediments were re-suspended in 0.25 mol/L sucrose (containing 5 mmol/L Tris, pH 7) from 100 μl of each of the re-suspended fresh and frozen sample pools, and centrifuged at 5000× g for 10 min, after which the cell pellet was rinsed 3 times with 0.25 mol/L sucrose. The sperm pellet 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), and 2% (w/v) dithiothreitol (DTT) in the presence of 1% (V/W) protease inhibitor cocktail (Sigma). Protein concentration was measured by the Bio-Rad Bradford protein assay using bovine serum albumin (Sigma) as a standard.

2.6. 2-DE

In total, 400 μg protein samples were resolved in loading buffer [8 mol L^{-1} urea, 2% CHAPS, 65 mmol L^{-1} DTT, 0.2% (w/v) Bio-Lyte

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