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Proteomic analyses of ram (*Ovis aries*) testis during different developmental stages

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ABSTRACT

Male reproductive capacity is essential for animal breeding and reproduction. In males, the testes produce sperm and secrete androgen, processes which require precise regulation by multiple proteins. The composition of proteins in the ram testes has not yet been studied systematically, thus, the application of proteomics to explore differential protein regulation during ram testes development is of great significance. In the present study, ram testes were studied at five different developmental phases to assess postnatal differences in protein regulation. Two dimensional electrophoresis (2-DE) was used to separate ram testes proteins at each developmental phase, yielding 45 different proteins, 37 of which were identified by Matrix Assisted Laser Desorption Ionization-Time of Flight-Time of Flight-Mass Spectrometry (MALDI-TOF/TOF-MS). Gene Ontology (GO) was used to specifically annotate the biological process, cellular composition, and molecular function of each identified protein. Most of the identified proteins were involved in structural formation, development, reproduction, and apoptosis of the testicular spermatogenic tissue and spermatozoa. Quantitative real time PCR (qRT-PCR), western blot and immunohistochemical methods were used to verify the proteins, and the results were consistent with that of 2-DE. The proteins that were different in abundance that were identified in this study can be used as biomarkers in future studies of ram reproduction.

Abbreviations: 2-DE, two dimensional electrophoresis; MALDI-TOF/TOF-MS, matrix assisted laser desorption ionization-time of flight- time of flight- mass spectrometry; GO, gene ontology; qRT-PCR, quantitative real time PCR; MS, mass spectrometry; TCA, trichloroacetic acid; IPG, immobilized pH gradient; SDS-PAGE, sodium dodecyl sulfate – polyacrylamide gel electrophoresis; ddH_2O, double-distilled H_2O; ACN, acetonitrile; TFA, trifluoroacetic acid; MALDI TOF-MS, matrix-assisted laser desorption/ionization time of flight flight-mass spectrometry; PMF, peptide mass fingerprint; MS/MS, tandem mass spectrometry; NCBI, national center for biotechnology information; ACTB, beta-actin; PVDF, polyvinyl difluoride; PBST, phosphate buffer solution/tween; RT, ~18–22 °C, room temperature; PRDX6, peroxiredoxin6; CNN2, calponin-2; PARK7, protein deglycase DJ-1; CFL1, cofilin-1; PBS, phosphate buffer solution; DAB, diaminobenzidine; ANOVA, one-way analysis of variance; eIF5A-1, eukaryotic translation initiation factor 5A-1; CA2, carbonic anhydrase 2; GLO1, lactoylglutathione lyase; TPI1, triosephosphate isomerase isoform X1; STMN1, stathmin; HSPB1, heat shock protein beta-1; ApoA-I, apolipoprotein A-I; RBP4, retinol-binding protein 4; EC-SOD3, extracellular superoxide dismutase [Cu-Zn] isoform X3; BTF3L4, transcription factor BTF3 homolog 4; TXN, thioredoxin; PGAM1, phosphoglycerate mutase 1; RPS12, ribosomal protein S12; GSTP, glutathione S-transferase P; FTH1, ferritin heavy polypeptide 1; COX5A, cytochrome c oxidase subunit 5A; TUBB, tubulin beta chain; PLEC, plectin isoform X13; TPM3, tropomyosin alpha-3 chain

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1. Introduction

Reproductive capacity is important in selecting rams in sheep production enterprises. The majority of ewes in some sheep breeds give birth to only a single lamb each year, so the reproductive performance of ewes, compared to other breeds, is relatively poor. In recent years, many studies have assessed the reproductive capacity of ewes, however, few studies have assessed the reproduction of rams, even though ram reproductive capacity is highly important for lamb production. The main reproductive organ of rams is the testes because it produces sperm and secretes testosterone (Wang, 2012) which are two important aspects of reproduction in males. These two functions interact and affect the capacity of rams to produce offspring. Studies of the testes have mainly focused on testicular histology, gene cloning and expression, transcriptomics, and microRNA selection and identification (Huang et al., 2011; Zhang et al., 2014; Pitia et al., 2015; Polguj et al., 2015). Previous studies have provided important information regarding testis tissue morphology and gene transcription, however, protein abundance, post-translational protein modification, and protein interactions have not been comprehensively studied.

In 1997, Cossio used two dimensional electrophoresis (2-DE) technology to separate proteins from the rat testis, after which Witzmann used the same method to separate proteins from the bull testes (Cossio et al., 1997; Witzmann et al., 1997). Researchers subsequently combined 2-DE with mass spectrometry (MS) to identify testicular proteins from humans, pigs, yaks, rats, and other species, yielding many proteins related to testicular development which have been used as biomarkers of male reproductive capacity (Huang et al., 2005; Paz et al., 2006; Li et al., 2011; Yang et al., 2016). No proteomic studies of the ram testis, however, have been reported. In the present study, therefore, proteomics assessments were applied to study changes in protein abundance during the development of the ram testis with the goal of identifying markers of male reproductive performance for utilization in future studies.

2. Materials and methods

2.1. Source of testis tissues

Animal care and experimental procedures were approved by and conducted in accordance with the Animal Committee Guidelines of the Gansu Agricultural University. Testes were from live purebred Small-tail Han rams at the Sanyang Sheep Breeding Farm (Baiyin, Gansu, China). Testicular samples were collected at 0 days, or 2, 5, 12, and 24 months of age (three animals in each age group). Both testes were removed, washed with sterile saline, and stored in liquid nitrogen and transported to the laboratory where these were stored at −80 °C. Part of each testis was fixed in 10% formalin in phosphate-buffered saline (Solarbio, BeiJing, China) for the immunohistochemical analysis.

2.2. Protein extraction

Total protein was extracted by trichloroacetic acid (TCA)/acetone (Bio-Rad, California, USA) precipitation using the method described previously (Lu et al., 2016). The quantified protein samples were stored at −80 °C prior to 2-DE analysis.

2.3. 2-DE assessments

The 2-DE assessment was performed as previously described (Lu et al., 2016). The samples were loaded onto IPG (Immobilized pH gradient) strips (Bio-Rad) and covered with mineral oil. After rehydration for 14 h at 50 V and 20 °C, isoelectric focusing was performed with the following programs: (S1) 200 V for 1 h; (S2) 500 V for 1 h; (S3) 1000 V for 1 h; (S4) gradient, 10,000 V for 5 h; (S5) 10,000 V for 9 h (90,000 Vh). After focusing, the IPG strips were equilibrated for 15 min in 10 mL of equilibration buffer I and II (Bio-Rad). The IPG strips were layered on top of vertical 12% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) for second-dimension separation using a PROTEAN II xi Cell system (Bio-Rad) at 15 °C. Separation was performed at 50 V for 1 h, followed by separation at 150 V until the bromophenol blue reached the bottom of the gel.

2.4. Protein visualization and image analysis

After electrophoresis, the polyacrylamide gels were visualized by overnight staining with Coomassie Brilliant Blue G-250 (Liu et al., 2015). Following staining, the gels were washed with double-distilled H_2O (ddH_2O, Solarbio) several times. Three gels were processed for each sample and assessed for relative abundance using a Powerlook 2100XL-USB image scanner (UMAX, Taiwan).

Protein spot analysis of the TIFF images was performed using PDQuest 8.0 software (Bio-Rad). Protein spots in gels were detected using PDQuest 8.0. Manual editing was conducted for some unidentified protein spots in geos and some impure spots were discarded. It is important to edit the spots using a uniform standard to avoid any effect of human factors affecting the experimental results. Background of the images was subtracted by removing the background in a non-spot mode. The process occurred by considering the pixel values around each protein spot. Standardization corrections were made for discrepancies caused by different sample preparations, loading methods, dyeing methods, or scanning methods. Protein spot auto-matching of the protein spots occurred using PDQuest 8.0 which allows for detection of overlapping spots on the three gels of each sample and combining of these spots on one of the gels as a reference gel. Comparisons among the different groups were made by matching the spots on the five reference gels, and calculating the relative volume by gray value of the spots. When the *t*-test value is $P < .05$, this was considered to be indicative of proteins that were in differential amounts. Spot filtering of selected protein spots from five different developmental stages were