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# Identification of differentially expressed proteins between bull X and Y spermatozoa

Xiaoli Chen[a,b,1], Huabin Zhu[a,1], Chengjiang Wu[c,1], Weidong Han[d], Haisheng Hao[a], Xueming Zhao[a], Weihua Du[a], Tong Qin[a], Yan Liu[a], Dong Wang[a,*]

[a]The Key Laboratory for Farm Animal Genetic Resources and Utilization of Ministry of Agriculture of China, Institute of Animal Science, Chinese Academy of Agriculture Sciences, Beijing 100193, China
[b]Animal Science and Technology College, Guangxi University, Nanning 530004, China
[c]Jilin Agriculture University, Changchun, 130118, China
[d]Research Center for Proteome Analysis, Key Laboratory of Systems Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, 200031, China

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ABSTRACT

Differential expression of genes leads to variation in phenotypes of X and Y sperm, even though some differential gene products are shared through an intercellular bridge. Differentially expressed proteins between X and Y sperm sorted from semen of nine bulls were compared using two-dimensional electrophoresis (2-DE) coupled with mass spectrometry (MS) analysis. Overall, 663±12 and 647±22 protein spots were detected in X sperm and Y sperm, respectively, and 42 significant protein spots were differentially expressed between them ($P<0.05$). Sixteen of these protein spots were successfully identified by MS and tandem MS and were found to be closely relevant to energy metabolism, stress resistance, cytoskeletal structure and the activity of serine proteases. Expression levels of two of these proteins, CAPZB and UQCRC1, were verified by Western blot. We propose that these differentially expressed proteins may affect the phenotype of X and Y sperm, binding and fusion of sperm/oocyte and development of the zygotic embryo. Our preliminary results provide an overview of differential expression in total protein levels between X and Y spermatozoa. Identification of these altered proteins may provide a theoretical basis for understanding the biological differences between the two types of sperm.



## 1. Introduction

Gender is an important aspect of animal husbandry production. For example, dairy cattle farmers prefer female cattle for milk production, while the bulls are selectively bred due to their greater genetic influence and economic importance than females. Sex selection in animal husbandry production can be achieved before insemination by sorting for X and Y chromosome bearing spermatozoa based on their unique characteristics. Previous reports have demonstrated significant differences in structure, morphology, motility and energy metabolism between X and Y sperm [1,2]. In particular, the differentially expressed proteins between these sperm can affect the binding and fusion of sperm and egg, as well as lead to variations in zygotic embryo development [3–5]. Therefore, detecting and analyzing the protein expression in X/Y sperm will not only guide our understanding of the differences in fertilization processes, initiation of embryonic development

* *Corresponding author*. Tel.: +86 10 62815892; fax: +86 10 13810509281.
E-mail address: dwangcn2002@vip.sina.com.cn (D. Wang).
[1] These authors contributed equally to this work.

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and formation of embryonic gender between these two types of sperm, but it can also offer new information and targets for development of efficient X/Y sperm sorting methods. However, there have been few studies on protein expression in X/Y sperm and even less in-depth analysis on differential protein expression between them [6,7]. The sperm sorting technology based on the variation of DNA content provides the basis for differential gene expression studies. The discovery of intercellular bridges by which sperm share materials has complicated the identification of differentially expressed proteins in X/Y sperm [5]. However, analysis of male transmission-ratio distortion (TRD) [4,8] demonstrated that some proteins are not shared between the two types of spermatozoa. In addition, anti-H-Y antibodies preferentially bind to either male bovine embryos [9] or sorted Y sperm of the bull [10], and gender-specific proteins have also been found in X sperm of mice [11]. At the same time, advances in proteomic technologies continue to provide new opportunities for analyzing subtle differences between X and Y sperm proteins [5,12].

In recent years, two-dimensional electrophoresis (2-DE) technology has been gradually optimized, and immobilized pH gradient (IPG) strips now provide a relatively stable separation system. The resolution and reproducibility of this method have been greatly improved, and up to 10,000 spots can be separated in one 2-DE gel [13]. The combination of 2-DE and mass spectrometry (MS) is especially effective in separating and identifying proteins. Proteomic technologies are widely being used in the study of sperm proteins with certain success. In total proteins from human sperm, 1397 spots have been detected [14], while more than 600 spots have been obtained from human sperm membrane proteins [15] using 2-DE technology. When combining 2-DE with MS, 98 sperm proteins were identified from 145 selected spots of total proteins extracted from human normozoospermic spermatozoa [16]. In another study, the proteins in asthenozoospermic samples were compared with the normal semen control by 2-DE analysis, and 17 differentially expressed proteins were identified using MS, which may be related to infertility in asthenozoospermia [17]. In this study, we selected the improved hot TRIzol method [18] to prepare proteins with high purity bovine X and Y spermatozoa, and the differentially expressed proteins were separated and identified using the combination of 2-DE and MS. By identifying and analyzing differences in the global protein expression between X and Y spermatozoa in this study, the ultimate objective was to uncover the products of differentially expressed genes not shared via the intercellular bridge. Our results may provide new information towards understanding the biological differences between the two types of sperm.

## 2. Materials and methods

### 2.1. Collection of semen samples and sorting

Semen samples were collected from nine randomly selected healthy, fertile Holstein bulls (2–3 years old) at the XY Breeding Livestock Co. (Tianjin, China). After analysis of sperm quality by microscopy to ensure the quality of the ejaculates (motility >80%, deformation ratio <15%), X and Y sperm were sorted immediately from the semen samples by flow cytometry at the XY Breeding Livestock Co., and purities were determined by PCR of the individually sorted sperm. According to the sequences of the bovine Amelogenin (AML) genes (gi: AB091790.1 for AMLY and gi: AB091789.1 for AMELX), a pair of sex-specific primers were designed with Oligo 6.0: AML395F, 5′-TTCTCACCAGTACCCTTCCTA-3′ and AML395R, 5′-TCAGAGGCAGGTCAGGAAGCA-3′ producing a 395-bp Y-chromosome band or a 458-bp X-chromosome band. The primers were synthesized by Invitrogen (Shanghai, China). The AML gene has two alleles, AMELY and AMELX, located on the X and Y chromosomes, respectively. There is a 63-bp deletion in the AMLY gene corresponding to the site from 6254 bp to 6316 bp in the AMLX gene sequence. The AML395 primers were designed to span the longer insertion sequence on the X chromosome. Because there is only one sex chromosome (either X or Y chromosome) in a single sperm, only one 458-bp band is detected for X sperm, while a single 395-bp band would be obtained from Y sperm. If no band is present, then sperm is not detected. Therefore, if the sex identification PCR is successful, the sex can always be identified according to the size of the single band detected, and there is no need for specific positive or negative controls for the PCR. The isolation of individual sperm and PCR identification were performed as detailed in our previous report [19]. Equivalent numbers of X and Y spermatozoa from three bulls were sampled and pooled. Thus, three X sperm sample pools and three Y sperm sample pools were obtained.

### 2.2. TRIzol-mediated protein preparation

X/Y spermatozoa ($3\times10^7$) from each sperm pool were used for protein preparation. Before protein preparation, all samples were washed in sucrose solution and deionized water. Each pellet containing $1.5\times10^7$ sperm was resuspended in 1 ml TRIzol (Invitrogen, Carlsbad, CA) and 20 μl β-mercaptoethanol (Sigma, St. Louis, MO). The following process for protein preparation was conducted according to Kirkland et al. [18] with some modifications. After 400 μl of isoelectric focusing (IEF) rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1 mM EDTA) and 5 mg dithiothreitol (DTT, GE Healthcare, Piscataway, NJ) were added to the pellet, 3 μl tributylphosphine (TBP, Bio-Rad, Richmond, CA) and 5 μl pH 4–7 linear IPG buffer was also added to the protein sample. The mixture was sonicated, and then insoluble materials were removed by centrifugation at 40,000×*g* for 45 min at 4 °C. Protein concentrations were determined using the Bradford assay.

### 2.3. 2-DE analysis

The protein solution was adjusted to 120 μg total protein in a volume of 450 μl, and three strips were loaded with three different X protein samples, while another three were loaded with three different Y protein samples. Therefore, a total of six 2-DE gels were run using the linear immobilized pH gradient (IPG) strips (pH 4–7 L, 24 cm,) (GE Healthcare). IEF was carried out at 20 °C for 80 kVh [30 V 12 h, 100 V 2 h, 200 3 h, 500 V 2 h, 1000 V 1.5 h, 10000 V 1.5 h, 10000 V (gradient) 20,000 Vh, 10,000 V 80,000 Vh], and the current limit was set as 50 μA