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Comparative transcript profiling of gene expression of fresh and frozen–thawed bull sperm

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ABSTRACT

Although frozen semen is widely used commercially in the cattle breeding industry, the resultant pregnancy rate is lower than that produced using fresh semen. Cryodamage is a major problem in semen cryopreservation; it causes changes to sperm transcripts that may influence sperm function and motility. We used suppression subtractive hybridization technology to establish a complementary DNA subtractive library, and combined microarray technology and sequence homology analysis to screen and analyze differentially expressed genes in the library, comparing fresh sperm with the frozen–thawed sperm of nine bulls. Overall, 19 positive differentially expressed unigenes were identified using microarray data and Significance Analysis of Microarrays software ($|\text{score (d)}| \geq 2$, fold change > 1, and false discovery rate < 0.05). Of 15 differentially expressed unigenes exhibited high sequence homology (E-value $\leq 1 \times 10^{-3}$), 12 were upregulated in frozen–thawed sperm, the remaining 3 were upregulated in fresh sperm, and 4 other clones were identified as unknown because of incomplete sequences or because there was no significant sequence homology (E-value $> 1E^{-03}$) and were considered novel genes. The expression of five of these genes—*RPL31*, *PRKCE*, *PAPSS2*, *PLP1*, and *R1G7*—was verified by quantitative real-time reverse transcription–polymerase chain reaction. There was a significant differential expression of the *RPL31* gene ($P < 0.05$). Our preliminary results provide an overview of differentially expressed transcripts between fresh and frozen–thawed sperm of Holstein bulls.

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1. Introduction

Frozen semen is widely used commercially in the cattle breeding industry, but the resultant rate of pregnancy is lower than that produced using fresh semen [1]. Although cryopreservation conditions have been optimized in many ways, such as the diluent used, freezing rate, and dose, the fertilization ability of sperm is persistently reduced by about seven times after cryopreservation, and sperm death and loss of motility cannot fully explain the reduction of fertilization ability [1,2]. It has been reported that sperm were damaged significantly during the cryopreservation cycle, and the cryodamage continued even in liquid nitrogen [3,4]. Earlier work has shown that medium quality semen samples (one freeze–thaw cycle) shared 98% and 39% of its RNAs with ideal (ejaculate) and poor-quality (three freeze–thaw cycles) samples, respectively. The results support the view that the population of spermatozoal RNAs varies rapidly in response to insult during each freeze–thaw cycle [5]. In the freeze–thaw cycle process, transcripts in the sperm were varied and affected fertilization accordingly. However, the authors only divided the sperm transcripts into groups resistant and sensitive to

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degradation, and could not analyze the resistance mechanism related to the differential transcripts further. In addition, related research on human sperm demonstrated that the amount of protamine 1 (*PRM1*) mRNA, associated with motility and capacitation, was significantly higher in low-motility sperm than in high-motility sperm [6]. Another study using complementary DNA (cDNA) microarray and quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR) verified that the expression of some genes related to sperm motility was higher in normal sperm than that in motility-impaired sperm [7]. These observations imply that transcriptional changes occur in sperm under certain conditions, and may affect fertilizing ability.

The suppression subtractive hybridization (SSH) technique is a simple and efficient means of generating cDNA that is highly enriched for differentially expressed genes of both high and low abundance in different species [8,9]. Combined SSH and microarray can be used to explore novel genes [10], and a study using this method verified the differing RNA profiles of bull sperm with different motility [11]. A recent study that used Illumina RNA-Sequencing reported on cryopreserved bovine spermatozoal transcription profiles, which included degraded and full-length nuclear-encoded transcripts and mitochondrial-encoded RNA. However, the authors did not remove nonmotile sperm from the samples, which may be the main reason degraded transcripts were present [12]. We aimed to discover the transcriptome variation related to the alterations in sperm fertility after cryopreservation. We hypothesized that freeze–thaw treatment would change the transcripts. Therefore, we used SSH combined with cDNA microarrays to identify differentially expressed genes between fresh and frozen–thawed bull sperm and verified the screening results by qRT-PCR. This study provides an experimental basis and theoretical foundation for research on the cryodamage mechanism and will provide an important reference for similar studies on swine, sheep, and other livestock.

2. Materials and methods

2.1. Semen collection and Pretreatment

The animal care and sample collection procedures in the present study were approved and conducted according to standards established by the Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing, China. Semen samples were obtained from nine random healthy fertile Holstein bulls (2–3 years old) from the Beijing Dairy Cattle Center (Beijing, China). Semen samples were collected using an artificial vagina (37 °C); sperm quality analyses were performed by microscopy to ensure the quality of the ejaculates (motility $>$ 80%; deformation ratio $<$ 15%). The semen samples of each animal were divided into two fractions: One was used as a fresh sample for RNA preparation and the other was cryopreserved using the conventional methods of the Breeding Bull Station of the Beijing Dairy Cattle Center. The main cryopreservation procedure was as follows: Extender (main components: sodium citrate, egg yolk, fructose, glycerol, penicillin, streptomycin) was added to the semen samples, which were then cooled slowly from 25 °C to 4 °C and allowed to equilibrate for 4 hours at 4 °C. The straws were cooled from 4 °C to −140 °C within 8 minutes, transferred to a liquid nitrogen tank, and stored. After the frozen samples had been stored for nearly 10 hours in liquid nitrogen, the straws were thawed in a 37 °C water bath for 30 seconds immediately before evaluation. Quality assessment of the frozen semen was carried out to ensure that sperm motility in each straw was greater than 35%, and the validated frozen semen was used for RNA preparation. Equivalent numbers of fresh and frozen–thawed sperm from each set of three bulls were pooled and divided into three pools each of fresh sperm and frozen–thawed sperm. Before RNA extraction, both fresh and frozen–thawed sperm sample pools were purified by centrifugation through a Percoll density gradient as described previously [13]. After washing with PBS, samples were incubated in somatic cell lysis buffer (0.1% SDS, 0.5% Triton X-100 in diethyl pyrocarbonate [DEPC]-treated water) on ice for 15 minutes to remove somatic cell contamination [5]. Then, they were washed twice in 0.25 mol/L sucrose solution diluted with DEPC-treated water (Invitrogen, Carlsbad, CA, USA) and centrifuged at 17,800×*g* at 4 °C for 10 minutes to pellet the purified sperm for RNA extraction. Both fresh and frozen–thawed sperm pellets were resuspended in PBS at the same sperm concentration and 3×10^7 fresh and frozen–thawed sperm from each semen pool were used for RNA extraction.

2.2. Hot TRIzol–mediated RNA isolation

Containing 1.5×10^7 sperm, each pellet was resuspended in 1 mL TRIzol (Invitrogen) and 40 μL β-mercaptoethanol (Sigma–Aldrich, St. Louis, MO, USA) and mixed vigorously. RNA isolation was conducted as described previously with some modifications [14]. After treatment with RNase-free DNase I (TaKaRa Bio Inc., Otsu, Japan), purified RNA pellets were redissolved in DEPC-treated water (Invitrogen) and stored at −80 °C. All RNA samples were quantified spectrophotometrically at 260 nm with an AstraGene Life Science Spectrophotometer (Astranet, Cambridge, UK). To test the integrity and examine for contamination from genomic DNA and somatic cell RNA, each RNA sample underwent PCR for the bovine glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*, NCBI Accession No. NC_007303) and CD45 antigen gene (*CD45*, NCBI Accession No. XM-599431). The upstream and downstream primers for the *GAPDH* gene were located at the fourth and sixth exons, respectively. All primer sequences are listed in Table 1. The PCR was as follows: Initial denaturation at 94 °C for 5 minutes, 33 cycles of denaturation at 94 °C for 30 seconds, annealing at 53 °C (*GAPDH* gene) or 52 °C (*CD45* gene), and extension at 72 °C for 30 seconds, and final extension at 72 °C for 10 minutes. PCR products were analyzed by 2% agarose gel electrophoresis.

2.3. SSH library construction and sequencing

Sperm RNA (500 ng) from the fresh and frozen–thawed sperm samples were used for RT with a Super SMART PCR

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