Comparative transcriptome analysis digs out genes related to antifreeze between fresh and frozen–thawed rooster sperm

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ABSTRACT The objective of this study was to investigate differences in mRNA expression between fresh and frozen–thawed sperm in roosters. In trial 1, gene expression profiles were measured using microarray with Affymetrix GeneChip Chicken Genome Arrays. The results showed that 2,115 genes were differentially expressed between the 2 groups. Among these genes, 2,086 were significantly downregulated and 29 were significantly upregulated in the frozen–thawed sperm group. Gene Ontology (**GO**) analysis showed that more than 1,000 differentially expressed genes (**DEG**) of all significantly regulated genes were involved in GO terms including biological processes, molecular function, and cellular component. Kyoto Encyclopedia of Genes and Genomes analysis showed that DEG were significantly ($P < 0.05$) enriched on ribosome, oxidative phosphorylation, proteasome, cell cycle, oocyte meiosis, and spliceosome pathways. In trial 2, ejaculated semen was collected from 18 roosters and divided into 5 recombinant HSP90 protein–supplemented groups (0.01, 0.1, 0.5, 1, or 2 μg/mL) and one control group with no recombinant HSP90 protein supplementation to evaluate the effect of recombinant HSP90 protein in the extender on post-thaw quality of rooster semen. The results showed that post-thaw sperm viability and motility was significantly improved ($P < 0.05$) in the extender containing 0.5 and 1 μg/mL of recombinant HSP90 protein compared with the control. Our preliminary results will provide a valuable basis for understanding the potential molecular mechanisms of cryodamage in frozen–thawed sperm and theoretical guidance to improve the fertility of frozen–thawed chicken sperm.

Key words: rooster, transcriptome, fresh and frozen–thawed sperm, recombinant HSP90 protein

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INTRODUCTION

Cryopreservation is an effective method for preserving germplasm, which greatly improves the utilization of excellent germplasm resources. It has broad application prospects in agriculture, aquaculture, and conservation of threatened species (Judycka et al., 2019; Silva et al., 2019; Ugur et al., 2019).

Frozen semen has been widely used in the cattle breeding industry. But sperm freezing for poultry is very limited because of its lower fertility and high individual within-breed variability (Blesbois, 2007; Chuaychu-Noo et al., 2016). However, sperm cryopreservation in poultry has a great potential of application in many fields, including artificial insemination practice, breeding selection, disease prevention, the conservation of genetic resources, and *ex situ* management of avian genetic resources (Thananurak et al., 2019; Thélie et al., 2019), which are of great economic importance for the commercial poultry industry. The cryopreservation of avian semen began in 1942 (Shaffner, 1942) but failed to achieve hatched chicks until 1949 (Polge et al., 1949). Although cryopreservation conditions have been optimized in many ways in the last decades, such as using the antioxidant (Najafi et al., 2019), glycerol (Rakha et al., 2017) or even different cooling rates (Madeddu et al., 2016), the fertilization ability of the sperm still persistently decreased after cryopreservation (Sule et al., 2013; Chen et al., 2015). This phenomenon may be attributed to high osmolality, ice crystal formation,

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mechanical harm, and pH changes (Cheng et al., 2015). However, most studies have mainly focused on exploring the effective cryoprotectants and optimizing the cryopreservation conditions, but focus on the antifreezing-related genes in chicken sperm has been obviously neglected. Spermatozoa contain a complex population of RNAs, including mRNA, interference RNA, antisense RNA, and microRNA (Polge et al., 1949; Hosken and Hodgson, 2014). Singh et al. (2016) analyzed chicken sperm transcriptome profiling by microarray analysis, and the results verified the existence of thousands of predominantly nuclear-encoded transcripts in chicken sperm. In the frozen–thawed cycle process, transcripts in the sperm varied rapidly in response to insult (Ostermeier et al., 2005). Chen et al. (2015) compared transcript profiling of gene expression of fresh and frozen–thawed bull sperm and identified 19 positive differentially expressed unigenes between fresh and frozen–thawed sperm of Holstein bulls. The result confirmed frozen–thawed treatment changed the transcripts.

Therefore, the purpose of this study was to identify differentially expressed genes (**DEG**) between fresh and frozen–thawed rooster sperm and to verify the screening results by quantitative PCR (**qPCR**). Meanwhile, we further studied the protective effect of the DEG on rooster semen cryopreservation by adding the recombinant protein of the DEG in the extender. This study will offer some new perspectives to improve the technology of chicken semen cryopreservation.

MATERIALS AND METHODS

Trial 1

This experiment was conducted to dig out genes related to antifreeze in the sperm by comparison of transcriptome profiles between fresh and frozen–thawed rooster sperm.

Sample Collection and Programming of Freezing and Thawing Semen Roosters had free access to diets and water in natural light cycle and natural temperature in the laboratory animal room of Beijing University of Agriculture. Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals. This study was approved specifically by the Animal Welfare Committee of Beijing University of Agriculture. Semen samples from 18 healthy fertile 6-month-old Hy-Line Brown roosters, selected from 30 roosters, were collected by the abdominal massage method (Quinn and Burrows, 1936). Semen samples of each bird were divided into 2 fractions: One was used as a fresh sample, and the other was used as a frozen–thawed sample. Before freezing, semen was diluted (1:1) using the Beltsville Poultry Semen Extender (**BPSE**) (pH = 7.4) consisted of sodium glutamate (0.867 g/100 mL), D-fructose (0.5 g/100 mL), potassium dihydrogen phosphate (0.065 g/100 mL), potassium hydrogen phosphate (1.27 g/100 mL), sodium acetate (0.26 g/100 mL), magnesium chloride hexahydrate (0.034 g/100 mL), citric acid potassium (0.064 g/100 mL), and water. All chemicals above were purchased from Sigma Chemical Co. (St. Louis, MO). Then, the semen was maintained at 5°C for 120 min. After addition of 6% N,N-dimethylacetamide (final concentration) to the BPSE diluent for 1 h at 5°C, the semen was frozen from 5°C to −35°C (the down rate of 7°C/min) and from −35°C to −120°C (the down rate of 9°C/min) and stored in liquid nitrogen finally. In the thawing process, the frozen semen was kept in a water bath evaporator at 37°C for 40 s.

Sperm Motility Assay The motilities of fresh and frozen–thawed semen from each bird were analyzed using a computer-assisted sperm analyzer system (WLJX-9000 Weili Color Sperm Analysis System, Weili New Century Science & Tech Dev., Beijing, China) and with settings adjusted to detecting avian sperm (Davila et al., 2015; Nabi et al., 2016). Five randomly selected microscopic fields were analyzed for each semen sample, and the mean value represented the sperm motility of samples.

Total RNA Isolation and Quality Assessment Before RNA extraction, equivalent numbers of fresh and frozen–thawed sperm from each set of 6 birds were pooled and divided into 3 sperm sample pools each of fresh and frozen–thawed sperm. Both fresh and frozen–thawed sperm sample pools were purified by density gradient centrifugation as described previously (Singh et al., 2016). After washing with phosphate-buffered solution, the samples were incubated in the somatic cell lysis buffer (0.1% sodium dodecyl sulfate, 0.5% Triton X-100 in diethyl pyrocarbonate–treated water) on ice for 30 min to remove somatic cell contamination (Ostermeier et al., 2005). Both fresh and frozen–thawed purified sperm pellets were resuspended in phosphate-buffered solution for RNA extraction.

The total RNA was isolated from fresh and frozen–thawed sperm sample pools using the TRIzol reagent (Ambion, Austin, TX) following the manufacturer's instructions. The RNA purity was evaluated by determining the A260/A280 and A260/A230 values and then visually confirmed through examining the 18S and 28S bands in a 1% agarose gel stained with ethidium bromide. The concentration of the total RNA extracted was measured using a Nanodrop spectrophotometer (Bibby Scientific Limited, Stone, UK). The extracted RNA was treated with Amplification Grade DNase I (Invitrogen, Shanghai, China) to remove potential DNA contamination. Each purified RNA extracted was divided into aliquots for use in Affymetrix GeneChip arrays (Affymetrix, Santa Clara, CA) and qPCR.

Microarray Workflow and Data Analysis Gene expression profiling of 6 samples, including 3 fresh sperm sample pools and 3 frozen–thawed sperm sample pools, was measured by microarray analysis. First, RNA of each sample was reverse transcribed to cDNA. First-strand cDNA was synthesized from RNA samples by reverse transcription reaction primed with the T7 oligo (dT) primer to synthesize cDNA containing a T7 promoter sequence. The obtained first-strand cDNA was then converted into double-stranded cDNA in the same reaction. Furthermore, the samples were then subjected to in vitro transcription to synthesize cRNA from the double-stranded cDNA templates using the T7 Enzyme

Mix (ABI, Shanghai, China). The cRNA was then purified to remove unincorporated NTPs, salts, enzymes, and inorganic phosphate using Qiagen RNeasy columns (Qiagen, Shanghai, China). Both concentration and the amount of cRNA were determined using the Nanodrop spectrophotometer.

The fragmentation of cRNA was carried out before hybridization. Microarray hybridization was carried out by Beijing Capital Bio Technology Co., Ltd. using the Affymetrix Chicken Genome Array (28,000 genes; Affymetrix). Hybridization was carried out for 16 h at 45°C in a rotary oven (Affymetrix); washing, staining (GeneChip Fluidics Station 450 [Affymetrix]), and scanning (GeneChip Scanner3000 [Affymetrix]) were carried out as per Affymetrix protocols (http://www.affymetrix.com/support/technical/manuals.affx). Expression Console software (Affymetrix) was used for image analysis and to determine probe signal levels (I, 2004).

Analysis of differentially expressed genes was performed using Significance Analysis of Microarrays software (CapitalBio, Beijing, China) (Ravikumar et al., 2009), and the selected level was as follows: P-value ≤ 0.05; with a fold change of 2 or a fold change ≤ 0.5. Gene Ontology (**GO**) enrichment analysis was performed for function corresponding to DEG in chicken using Molecule Annotation System version 3.0 (CapitalBio; http://bioinfo.capitalbio.com/mas3/) (Ashburner et al., 2000). The significant threshold value for GO term enrichment was the false discovery rate less than 0.05. Kyoto Encyclopedia of Genes and Genomes (**KEGG**) pathway enrichment analysis (Kanehisa and Goto, 1999) with DEG was performed using DAVID version 6.7 (http://david.abcc.ncifcrf.gov/) (Huang et al., 2008).

Quantitative Real-Time PCR Analysis To validate the microarray assay results, 4 DEG were selected for qPCR assays using the CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) and Superreal Premix Plus (SYBR Green, Tangent Biotech (Beijing) Co., Ltd., Beijing, China). Chicken glyceraldehyde 3-phosphate dehydrogenase was chosen as an endogenous control to correct for analytical variations. The primers used are listed in Table 1. To calculate the mRNA expression of selected DEG, the ΔCt values were used for detection of their mRNA related to the internal control glyceraldehyde 3-phosphate dehydrogenase expression using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The significance of gene expression difference between the 2 groups was assessed using the t test of SPSS 22.0 (SPSS Inc., Chicago, IL). All genes marked as significant exhibited $P < 0.05$ or $P < 0.01$.

Trial 2

We evaluated the protective role of recombinant HSP90 protein (Sino Biological Inc., Beijing, China) on rooster sperm during cryopreservation. Recombinant HSP90 protein was added to BPSE at a concentration of 0 (control), 0.01, 0.1, 0.5, 1, or 2 μg/mL. Post-thaw sperm motility was analyzed using a computer-assisted sperm analyzer system as mentioned previously. Post-thaw sperm viability was evaluated using the eosin–nigrosin stain method as we described previously (Qi et al., 2019). In brief, sperm suspension smears were prepared by mixing 10 μL of the sperm sample with 20 μL of stain and assessed by counting 200 cells using an Olympus BX51 microscope (Olympus Co., Tokyo, Japan) at a final magnification of 400×. The unstained sperm is considered alive, whereas the purple sperm is considered dead.

Statistical Analysis All data were analyzed using SPSS 22.0. The significance of the difference in sperm motility between fresh and frozen rooster semen was assessed using the t test by SPSS 22.0 for Windows. One-way ANOVA followed by Duncan's multiple comparison test was used to examine statistical differences among recombinant HSP90 protein treatments. Statistical significance was defined at $P < 0.05$.

RESULTS

Comparison of Transcriptome Profiles Between Fresh and Frozen–Thawed Rooster Sperm by Microarray Analysis

The data of fresh and frozen–thawed rooster semen showed that the frozen–thawed treatment significantly decreased the motility by 51.50% (78.55 ± 4.50% vs. 27.05 ± 1.22%; respectively, $P < 0.05$) using BPSE. Gene expression microarray analysis revealed 38,535 probes in all samples (Supplementary Table 1). The majority of chicken sperm transcript probes (73.73%) were annotated in gene symbol. To identify the molecular mechanism of cryopreserved semen, the transcriptome

Table 1. The specific primers used for qPCR in this study.

Gene	Sequences	Product size	Accession no.
GAPDH	F-AGAACATCATCCCAGCGTCCA R- CAGGTCAGGTCAACAACAGAG	131 bp	NM_204305.1
CIRBP	F- CCAGAGGAGGTGGAGACAGA R- AGCCACCTTGACTCCTGCTA	105 bp	NM_001031347.1
RHOA	F- GAAGCAGGAGCCTGTCAAAC R- GCAGCTCTAGTGGCCATTTC	132 bp	NM_204704.1
HSP70	F- TTGATAAGGGCCAGATCCAG R- TGTTCAGCTCTTTGCCATTG	105 bp	NM_001006685.1
HSP90	F- CAAGCCTATTTGGACCAGGA R- CAAGTGGTCCTCCCAGTCAT	94 bp	NM_001109785.1

Abbreviations: CIRBP, cold-inducible RNA-binding protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; qPCR, quantitative PCR; RHOA, Ras homolog family member A.

profiles of fresh and frozen–thawed rooster semen were compared by gene microarray analysis. Scatter plots enabled us to identify genes whose expression level underwent at least a 2-fold change upon treatment between the groups. There were 2,115 DEG in the 2 experimental groups. Among these genes, 2,086 were significantly downregulated, and 29 were significantly upregulated in the frozen–thawed sperm group (Figure 1). The *CIRBP*, *RHOA*, *HSP70*, and *HSP90* genes from DEG were selected for qPCR analysis and correlated with the gene microarray results (Figure 2). These genes were chosen based on their biological function related to antifreeze, and on microarray analysis, it was found these genes were significantly downregulated in the frozen sperm group. On qPCR analysis, all selected genes were found to be significantly differentially expressed between the 2 groups ($P < 0.05$). The tendency for differential expression of each gene was consistent with the microarray and qPCR results. The gene expression fold changes measured by the microarray and qPCR methods were highly correlated with a significant determination of 0.84 ($P < 0.05$).

Gene Ontology analysis was performed to identify the obviously altered biological functions of those genes differentially expressed between the 2 groups. The results showed that more than 1,000 DEG of all significantly regulated genes were involved in GO terms including biological processes (**BP**), molecular function (**MF**), and cellular component (Supplementary Table 2). The Gene Ontology term of BP (false discovery rate < 0.05) affected by DEG was mostly associated with translation, modification-dependent protein catabolic process, modification-dependent macromolecule catabolic process, proteolysis involved in cellular protein catabolic process, and so on. Based on GO classification of the cellular component, these genes were grouped into main functional clusters: ribosome, cytosol, organelle envelope, mitochondrial envelope, and so on. There were also several GO terms enriched significantly for MF such as structural constituent of ribosome, structural molecule activity, translation initiation factor activity, and translation factor activity, nucleic acid binding. The detailed information of GO terms is shown in Table 2. The KEGG database was used to identify those signaling pathways influenced by DEG (Supplementary Table 3). As per the results, there were 6 signaling pathways ($P < 0.05$) that were involved: ribosome, oxidative phosphorylation, cell cycle, proteasome, spliceosome, and oocyte meiosis pathways (Figure 3).

The Protective Effect of Recombinant HSP90 Protein on Rooster Sperm During Cryopreservation

Before freezing, sperm motility was 80.53 ± 1.29% (mean ± SEM). Effects of different levels of recombinant HSP90 protein in the extender on post-thaw sperm

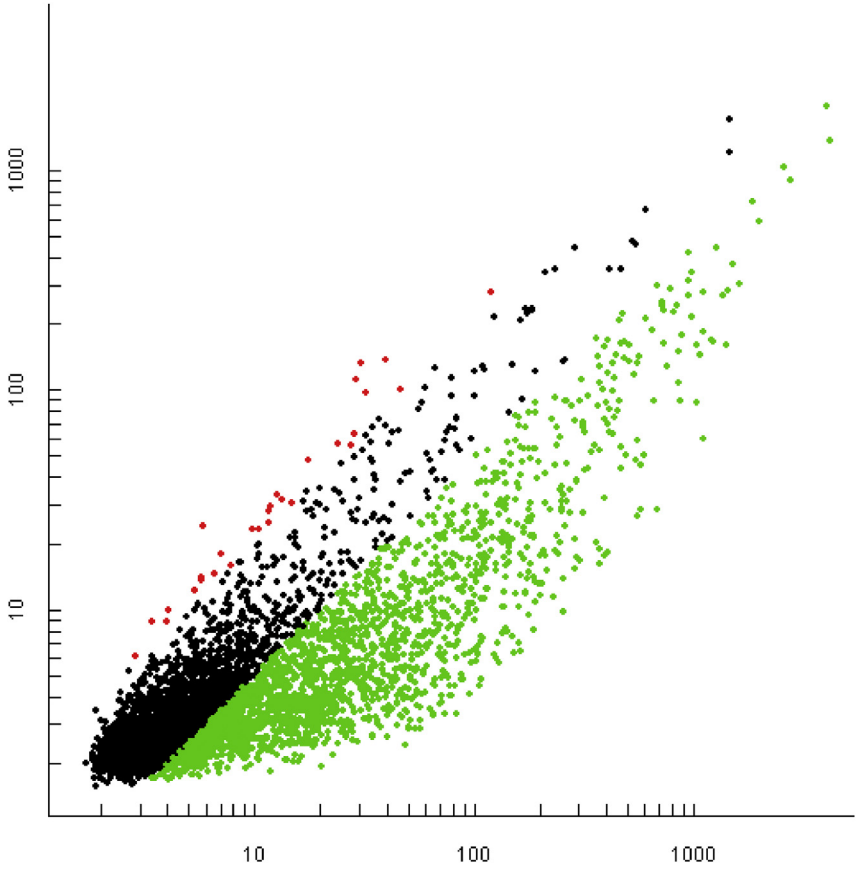

Figure 1. Gene expression level in the fresh and frozen–thawed rooster semen groups. The x-axis plots gene expression counts in the fresh group, and the y-axis plots gene expression counts in the frozen–thawed group. The red points indicate genes with significant upregulation, green points indicate genes with significantly downregulation, and black points indicate genes with insignificant difference.

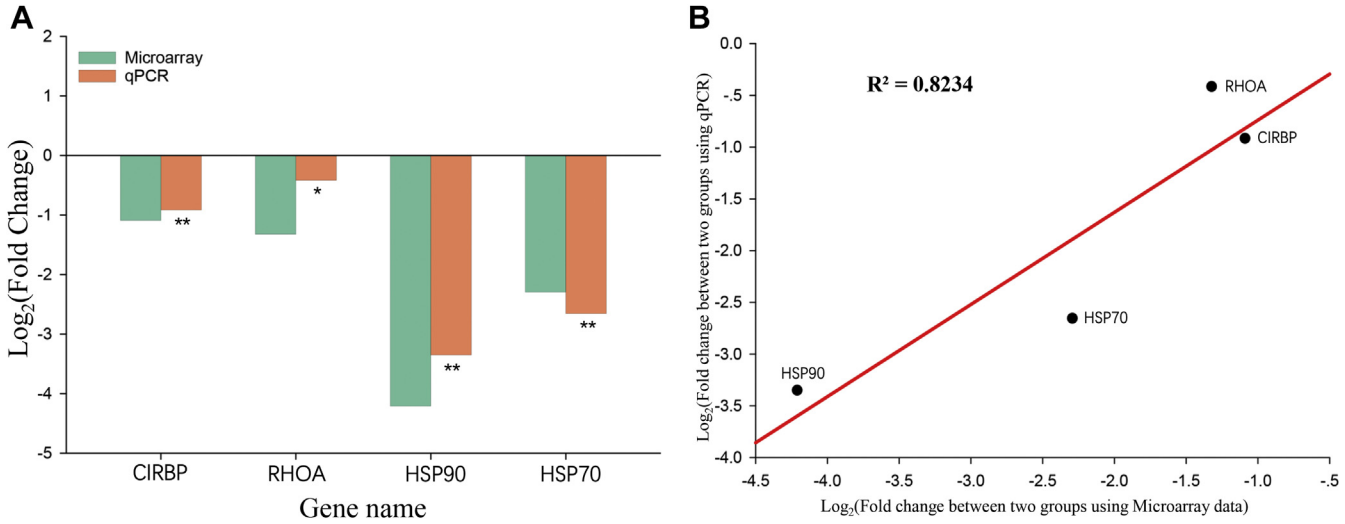

Figure 2. Validation of genes expression patterns obtained by microarray and qRT-PCR analysis. (A) Log_2 fold changes (FC) assessed by PCR and microarray for 4 genes. Asterisks on the qPCR values indicate significant differences between the 2 groups (*$P < 0.05$, **$P < 0.01$). (B) The relationship between the log_2FC between the 2 groups using microarray and qRT-PCR data ($P < 0.05$). CIRBP, cold-inducible RNA binding protein; qPCR, quantitative PCR; RHOA, Ras homolog family member A; RT, real time.

viability are presented in Figure 4. Post-thaw sperm viability was significantly improved ($P < 0.05$) in the extender containing 0.5 and 1 μg/mL recombinant HSP90 protein compared with the control, and the sperm viability in the 0.5 μg/mL group was higher than that in any other groups ($P < 0.05$). No significant difference in viability of post-thaw sperm was observed in the extenders with 0.01, 0.1, and 2 μg/mL

Table 2. Gene Ontology terms enriched significantly (FDR < 0.05) in this study.

Category	GO ID	GO term	Gene number	FDR
BP	GO:0006412	Translation	81	6.91E-28
	GO:0019941	Modification-dependent protein catabolic process	39	1.44E-06
	GO:0043632	Modification-dependent macromolecule catabolic process	39	1.44E-06
	GO:0051603	Proteolysis involved in cellular protein catabolic process	41	3.77E-06
	GO:0044257	Cellular protein catabolic process	41	6.12E-06
	GO:0030163	Protein catabolic process	43	8.30E-06
	GO:0044265	Cellular macromolecule catabolic process	46	1.74E-05
	GO:0009057	Macromolecule catabolic process	49	3.69E-05
	GO:0006091	Generation of precursor metabolites and energy	38	3.96E-05
	GO:0006511	Ubiquitin-dependent protein catabolic process	27	5.73E-05
	GO:0007017	Microtubule-based process	26	3.67E-02
	GO:0006119	Oxidative phosphorylation	18	3.85E-02
CC	GO:0005840	Ribosome	60	1.35E-25
	GO:0030529	Ribonucleoprotein complex	70	3.75E-19
	GO:0043228	Nonmembrane	153	1.09E-14
	GO:0043232	Intracllular nonmembrane-bounded organelle	153	1.09E-14
	GO:0005829	Cytosol	46	1.48E-09
	GO:0031967	Organelle envelope	45	1.12E-04
	GO:0044445	Cytosolic part	19	1.42E-04
	GO:0031975	Envelope	45	1.65E-04
	GO:0005740	Mitochondrial envelope	35	1.39E-03
	GO:0033279	Ribosomal subunit	14	2.87E-03
	GO:0005739	Mitochondrion	63	4.27E-03
	GO:0015935	Small ribosomal subunit	10	1.65E-02
	GO:0031966	Mitochondrial membrane	30	4.20E-02
	GO:0044429	Mitochondrial part	36	4.54E-02
MF	GO:0003735	Structural constituent of ribosome	57	1.76E-26
	GO:0008135	Translation factor activity, nucleic acid binding	22	2.93E-06
	GO:0005198	Structural molecule activity	70	4.27E-06
	GO:0003743	Translation initiation factor activity	16	5.80E-04

Abbreviations: BP, biological process; CC, cellular component; FDR, false discovery rate; GO, Gene Ontology; MF, molecular function.

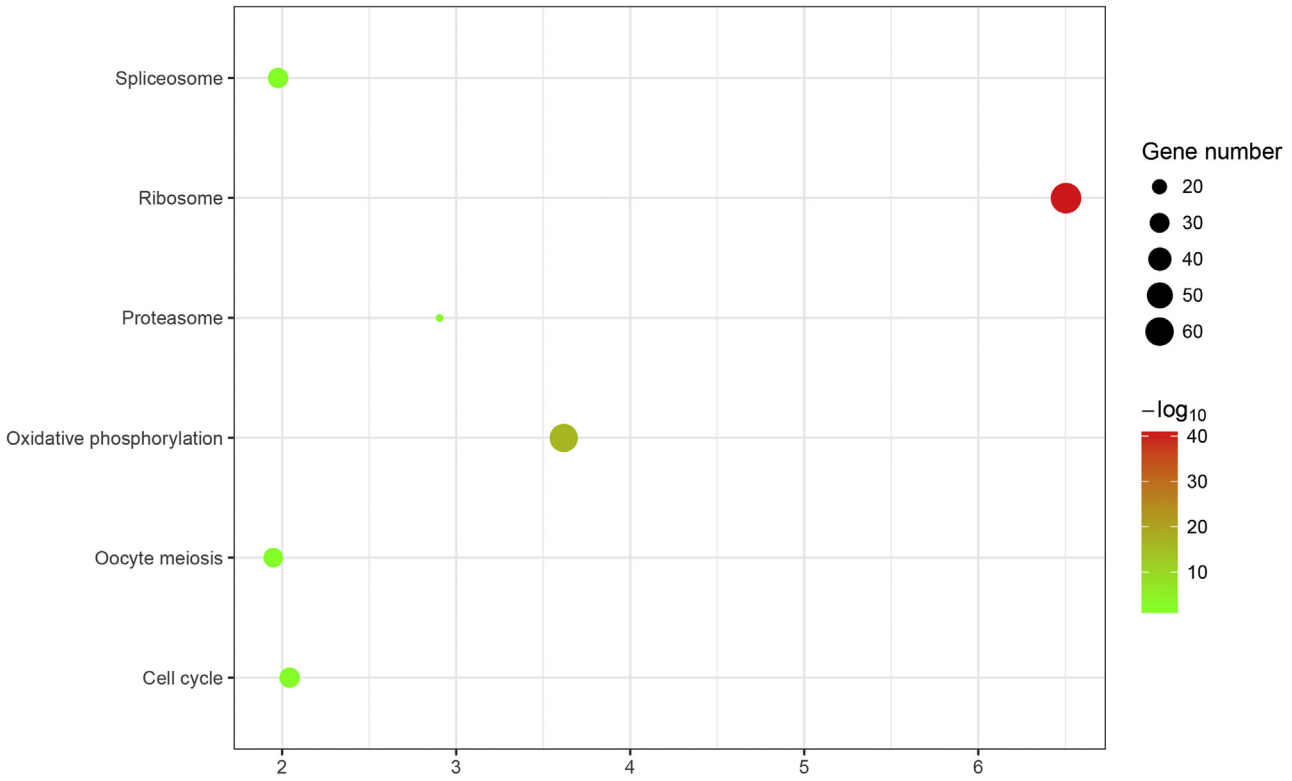

Figure 3. Scatter plot of those KEGG pathways with significant enrichment. The x-axis represents the fold enrichment. The y-axis is the pathway with significant enrichment. *P*-value represents the corrected P by Benjamini, and a small *P* value indicates high significance. KEGG, Kyoto Encyclopedia of Genes and Genomes.

recombinant HSP90 protein and in the control. Effects of different levels of recombinant HSP90 protein in the extender on post-thaw sperm motility are presented in Figure 5. Post-thaw sperm motility was dramatically enhanced ($P < 0.05$) in the extender containing 0.5 and 1 μg/mL recombinant HSP90 protein compared with the control, and the sperm motility in the 0.5 μg/mL group was higher than in any other groups ($P < 0.05$). No significant difference in motility of post-thaw sperm was observed in the extenders with 0.01, 0.1, and 2 μg/mL recombinant HSP90 protein and in the control.

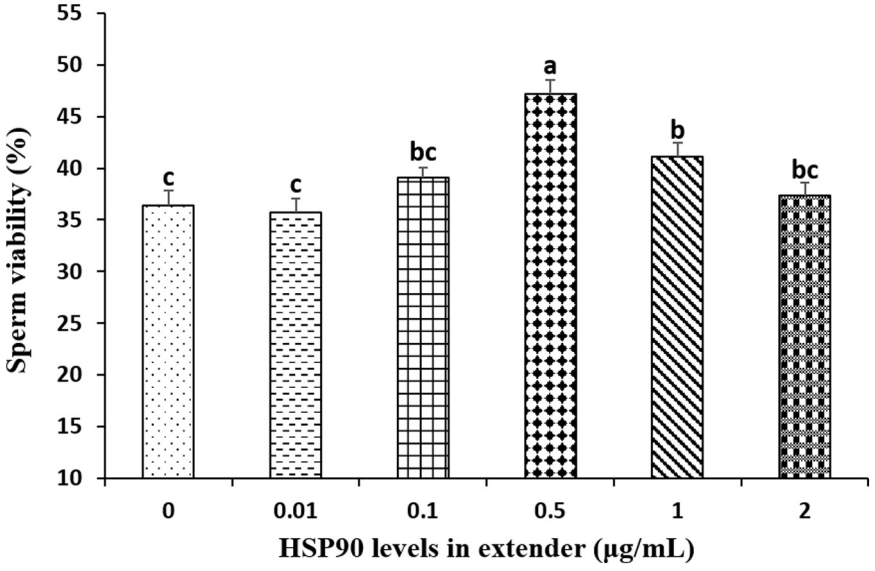

Figure 4. Viability of the post-thaw sperm with addition of recombinant HSP90 protein at different levels of 0, 0.01, 0.1, 0.5, 1, and 2 μg/mL. Data are expressed as mean ± SE of 6 replicates per treatment. Means without a common letter differ ($P < 0.05$).

DISCUSSION

In the present study, a significant difference in sperm motility between the fresh and frozen–thawed groups was observed (78.55 ± 4.50% vs. 27.05 ± 1.22%, respectively). Based on transcriptome microarray analysis, we verified 2,115 DEG in the 2 experimental groups. Among these genes, 2,086 were significantly downregulated, and 29 were significantly upregulated in the frozen–thawed sperm group. Historically, the sperm has been thought to be a highly differentiated cell without organelles, and gene expression does not occur in a mature sperm. A mature sperm is only a carrier for transferring male genomes to the oocyte (Boerke et al., 2007). However, recent research has shown that the sperm delivers more to the oocyte than just the paternal haploid genome (Singh et al., 2016). On fertilization, the sperm provided a complete and highly structured genome and offered a variety of divergent functions (Krawetz, 2005). The results of this study also confirmed the presence of mRNA expression in the poultry sperm. It is noteworthy that the number of genes detected in the sperm is only about 7% of the total, indicating that the level of mRNA in poultry sperm is extremely low, similar to that of mammals (Miller et al., 1994). In this study, DEG were involved in a variety of functions and roles in cell components, MF, and BP, suggesting that the freeze–thaw process affects sperm gene expression in many ways.

Energy metabolism is a key factor supporting sperm functions (Piomboni et al., 2012). Sperm motility has been found to be enhanced by increasing the activity of mitochondrial respiratory enzyme activity and ATP production through upregulation of the mRNA expression of related enzymes in roosters (Kamali Sangani et al., 2017; Jiao et al., 2018). Analysis of chicken sperm transcriptome by microarray analysis in the present study showed that many DEG significantly enriched in the oxidative phosphorylation pathway, such as NADH dehydrogenase, ubiquinol-cytochrome c reductase, ATP synthase, cytochrome c oxidase, and succinate dehydrogenase, were markedly downregulated. In mammals, glycolysis is the main source of energy metabolism in the sperm in an aerobic state (Singh et al., 2011). However, this condition differs in avian species. The mitochondria in the middle of the sperm provide energy for capacitation, acrosome reaction, and maintenance of motility in roosters (Madeddu et al., 2010). The results of the present study suggested that the mRNA expression of mitochondrial respiratory chain enzymes in chicken sperm was affected by frozen–thawed treatment and might lead to a decrease of ATP production, resulting in the block of the energy source for sperm motility. The mechanism underlying warrants further study.

In the current research, 58 ribosomal protein genes were differentially expressed between fresh and frozen–thawed sperm and significantly enriched in the ribosome pathway as per the KEGG analysis. Ribosomal proteins might play an important role in spermatogenesis and/or fertilization (de Mateo et al., 2011). Ribosomal protein L31 (RPL31) located in the 60S ribosomal subunit was markedly upregulated in frozen–thawed bull sperm (Chen et al., 2015), which indicated that it might play an important role in sperm cryopreservation. Ribosomal protein L29 (RPL29) existing in a variety of tissues is a constituent of membrane-associated and cytoplasmic translationally active ribosomes, which regulates the efficiency of protein synthesis (Kirnsafran et al., 2010). Research showed that loss of the *RPL29* gene invariably resulted in low sperm motility in murine sperm (Aravindan et al., 2014). Ribosomal proteins were also found in an isolated human sperm nucleus, suggesting that they might play a vital role in spermatogenesis and/or fertilization (de Mateo et al., 2011). In our study, both *RPL31* and *RPL29* mRNA expression were

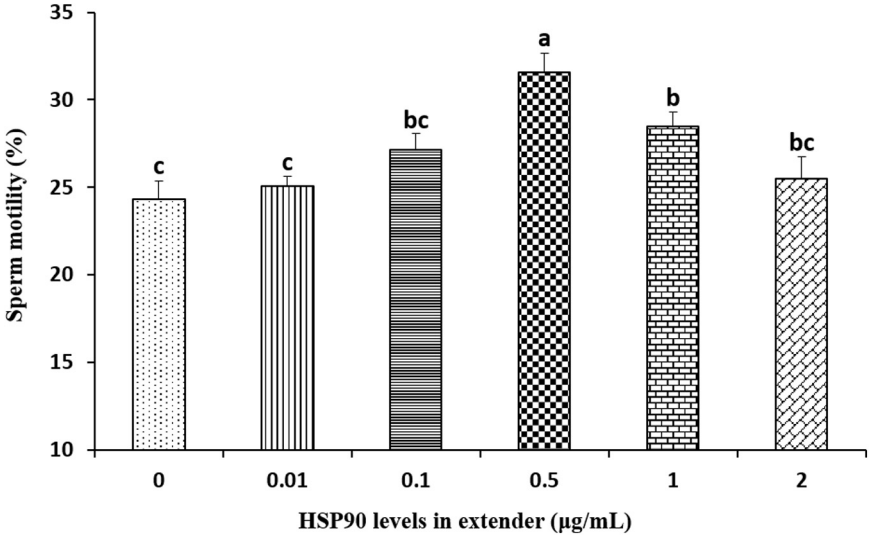

Figure 5. Motility of the post-thaw sperm with addition of recombinant HSP90 protein at different levels of 0, 0.01, 0.1, 0.5, 1, and 2 μg/mL. Data are expressed as mean ± SE of 6 replicates per treatment. Means without a common letter differ ($P < 0.05$).

significantly decreased by frozen–thawed treatment in rooster sperm, which may be a result of cold stress and requires further study.

Spermatogenesis is a complex process of cellular differentiation. Cell cycle regulatory genes play an important role in spermatogenesis. The cyclins and their cyclin-dependent kinase partners, the CDKs, are key regulators of the cell cycle (Wolgemuth, 2011; Wolgemuth et al., 2013). Lack of *cyclin A1* leads to cell arrest in the late diplotene stage of the meiotic cell cycle, resulting in an interruption of spermatogenesis and male sterility (Wolgemuth, 2011). In the present study, 28 DEG were significantly enriched in the cell cycle pathway, including *cyclin A1*, *cyclin H*, *cyclin B2*, *CDK1*, and *CDKN1B*. The result suggests that these genes are not only involved in cell cycle regulation but also selectively retained in the mature sperm and may be associated with the sperm's ability to resist freezing. Whether the decreased levels of cell cycle regulation genes cause the decrease of chicken sperm motility after frozen–thawed treatment requires further investigation.

The spliceosome, a huge and dynamic ribonucleoprotein complex, regulates the process of pre-mRNA into mRNA (Wahl et al., 2009). The latest research showed that the spliceosome core components were involved in human reproductive disease. Spliceosome dysregulation damaged the differentiation of spermatogonia, abolishing the maturation of germ cells into a sperm (Wu et al., 2016). Further research found that the spliceosome component SNRPA1, which belongs to the U2 snRNP A family, was essential for male fertility (Wu et al., 2016). Lack of SNRPA1 does result in the accumulation of spermatogonia that fail to differentiate into a mature sperm (Wu et al., 2016). Our data showed a significant decrease in the *SNRPA1* mRNA level after frozen–thawed treatment. Whether the decrease of *SNRPA1* causes the decrease of chicken sperm motility requires further investigation.

In addition, the DEG *CIRBP* and *RHOA* may also play an important role in rooster sperm cryopreservation. Cold-inducible RNA-binding protein (CIRBP) is a conserved cold stress protein that participates in a variety of signal transduction pathways required for cell function (Liu et al., 2019), which can be induced after exposure to a moderate cold shock in different species (Zhong and Huang, 2017). *CIRBP* is expressed in primary spermatocytes, secondary spermatocytes, and round spermatids, but it is not expressed in Sertoli cells or Leydig cells. CIRP-deficient mice showed a reduction in the number of undifferentiated spermatogonia, which indicates that *CIRBP* may have a role in spermatogenesis (Masuda et al., 2012; Zhong and Huang, 2017). It has been reported that CIRBP has a protective effect on the survival and apoptosis of germ cells (Zhong and Huang, 2017). However, *CIRBP* upregulation was observed only in the case of mild but not severe hypothermia or cold stress (Wang et al., 2015). In the mouse germ cell line, the mRNA expression of *CIRBP* was increased when culture temperature was reduced from 37°C to 32°C (Nishiyama et al., 1998). In our study, a significant decrease in the *CIRBP* mRNA level was observed after frozen–thawed treatment, which indicates that *CIRBP* will be downregulated under severe cold stress. The mechanism of the *CIRBP* gene in the process of cryopreservation needs further study. Ras homolog family member A (RHOA) has been reported to be involved in many BP, including participating in the regulation of the actin cytoskeleton (Wang et al., 2009), contributing to the pathologic processes of cancer (Wu et al., 2010), promoting hypoxia-induced angiogenesis (Ma et al., 2012), regulating chronic rejection of cardiac allografts (Liu et al., 2017), and so on. Our previous research proved that RHOA was related to the antifreeze ability of mouse embryos. Embryo cryopreservation downregulated the expression of RHOA both in real-time PCR and Western blot analysis (Gu et al., 2017). Similarly, the mRNA expression of RHOA was significantly downregulated by frozen–thawed treatment in the present study (fold changes = 6.56). The connections between the role of RHOA and cryopreservation of rooster sperm need to be elucidated.

The 70-kDa heat shock protein (HSP70), a member of the heat shock protein family, is also involved in the regulation of reproductive system development and function (Nixon et al., 2017).The 70-kDa heat shock protein 2 (HSPA2) has been shown to be critical for the progression of germ cell differentiation during spermatogenesis in the mouse model (Nixon et al., 2017). Adding 70-kDa heat shock protein 8 (HSPA8) to the media used for cryopreserving the sperm could improve the quality of brown bear sperm after cryopreservation (Alvarez-Rodríguez et al., 2013). A similar result was also observed in bull sperm cryopreservation (Holt et al., 2015). In addition, it has been shown that HSPA8 could improve the sperm viability at body temperature in vitro in boars, bulls, and rams (Elliott et al., 2009; Lloyd et al., 2012). In the present study, the gene expressions of *HSPA2* and *HSPA8* were significantly downregulated by frozen–thawed treatment. The result indicates that *HSPA2* and *HSPA8* may play a critical role in rooster sperm cryopreservation.

The 90-kDa heat shock protein (HSP90AA1 [HSP90]), a member of the heat shock protein family, has been found localized in the sperm tail in all species examined and plays a critical role in mediating sperm fertility (Wang et al., 2014). On microarray and qPCR analysis, our study showed that the mRNA expression of *HSP90* exhibited highly significant difference between the 2 groups. Expression of *HSP90* was higher in fresh sperm than in frozen–thawed sperm (fold changes = 18.51). It was consistent with the previous study that the level of *HSP90* expression in the sperm was significantly reduced after cryopreservation (Cao et al., 2003; Zhang et al., 2015). The reason might be due to cryodamage to the structure and function of the sperm during the freezing–thawing process, which inhibited the gene expression of *HSP90*. Whether adding HSP90 protein in the extender can improve sperm antifreeze ability is unclear. In trial 2, we evaluated the protective role of recombinant HSP90 protein on rooster

sperm during cryopreservation. The results showed that with the increase of recombinant HSP90 protein levels in the extender, the viability and motility of post-thaw sperm increased quadratically and reached the maximum value with addition of .5 μg/mL of recombinant HSP90 protein. Similar findings were also reported in buffalo bull sperm cryopreservation by Qadeer et al., 2014. It has been shown that cold stress can cause irreparable damage to ATP generation (Aramli, 2014). HSP90 protein is involved in the metabolic process of ATP (Li and Buchner, 2013). We speculate that HSP90 protein may play a role in sperm cryopreservation by blocking ATP synthesis and reducing intracellular energy consumption. In addition, HSP90 protein may protect the sperm by activating nitric oxide synthase to produce nitric oxide and eliminating reactive oxygen species produced during sperm cryopreservation (Garcia-Cardena et al., 1998). The underlying mechanism by which HSP90 protein improves the viability and motility of post-thaw sperm remains to be further studied.

In conclusion, the DEG identified between fresh and frozen–thawed rooster sperm are involved mainly in the ribosome, oxidative phosphorylation, cell cycle, and spliceosome pathways. The majority of genes involved in rooster sperm motility are localized in the ribosome, organelle envelope, cytoplasm, and mitochondrion. Supplementation of recombinant HSP90 protein in the extender at a concentration of 0.5 μg/mL improved the viability and motility of the cryopreserved rooster semen. Future studies are required to verify the effect of other identified genes (e.g., *SNRPA1*, *RPL29*, *CIRBP*, *RHOA*, *HSP70*) on the efficiency of sperm cryopreservation.

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SUPPLEMENTARY DATA

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