doi: 10.17221/8239-CJAS

Association of *heat shock protein 70* with motility of frozen-thawed sperm in bulls

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ABSTRACT: *Heat shock protein 70* (*HSP70*) is considered as a gene which affects semen quality traits. The present study attempted to investigate the relationship between the *HSP70* expression level and motility of bull sperm during the process of freezing-thawing. Semen samples were collected from 5 QinChuan bulls by artificial vagina. Sperm motility and plasma membrane integrity of the semen samples at three stages (fresh, after equilibration, and frozen-thawed) were evaluated. The *HSP70* expression level at the three stages was detected using real-time PCR. The results indicated that *HSP70* expression level, membrane integrity, and sperm motility in the fresh semen were higher than those of the sperm after equilibration and freezing-thawing ($P < 0.05$), the *HSP70* expression level, plasma membrane integrity, and sperm motility in sperm after equilibration were higher than those of the frozen-thawed sperm ($P < 0.05$). The correlation between *HSP70* expression level and sperm motility was positive (ranging from 0.327 to 0.785). The results suggest that *HSP70* expression level in bull spermatozoa was gradually decreased following the process of freezing-thawing, and might be associated with bull sperm motility.

Keywords: gene expression; cryopreservation; *HSP70*; sperm motility

INTRODUCTION

Artificial insemination (AI) is now widely used in cattle breeding. Frozen-thawed bull semen plays an important role in this procedure. However, there is about 40–50% loss in bull semen quality during the freezing-thawing process, including sperm motility, effective survival time, and antioxidant enzymes activity (Hu et al. 2011). Various extenders and freezing procedures have been described in bull semen to minimize detrimental effects of cryopreservation on sperm. However, sperm analysis is mainly focused on sperm parameters (motility, viability, membrane integrity, acrosome integrity etc.). The mechanism which affects frozen-thawed sperm quality at molecular level has not been thoroughly investigated.

Some proteins in spermatozoa are associated with mitochondrial energy production (Ruiz-Pesini et al. 1998), nitric oxide production (Lewis et al. 1996), and protein phosphorylation (Vijayaraghavan et al. 1996). The process of freezing-thawing could cause damage to the function of sperm proteins (Cao et al. 2003; Martin et al. 2007). Previous study suggested that some proteins also play important roles in the maintenance of sperm motility during the process of freezing-thawing (Huang et al. 1999). Therefore, analyzing the change of protein in sperm could be a useful method to predict sperm quality. Heat shock proteins (HSPs) are a group of proteins which protect cells against apoptosis during injury and oxidative stress (Beere and Green 2001). Among the HSP family, HSP70 could maintain protein conformation, stabilize unfolded precursor proteins prior to the assembly into macromolecular complexes, and participate in transfer proteins across intracellular membranes (Gething and Sambrook 1992). HSP70 appeared to play an important role in sperm function after ejaculation and it was identified in proteins extracted from bull (Kamaruddin et al. 1996) and boar sperm (Huang et al. 2000). However, the correlation

Supported by the 12th Five-Year Plan Rural Areas State Science and Technology Support Projects (2011BAD19B04-3) and the Basic Scientific Research Expense of Sci-Tech Innovation Major Project of Northwest A&F University (QN2011061).

doi: 10.17221/8239-CJAS

between the *HSP70* expression level and the sperm motility following the process of freezing-thawing in bulls has not been studied thoroughly.

Therefore, owing to the ability of HSP70 to protect cells against stress, the objective of the present study was to determine whether or not *HSP70* expression level in bull sperm changes following the process of freezing-thawing. This work also investigates the relationship between *HSP70* expression level and motility of frozen-thawed bull sperm.

MATERIAL AND METHODS

Chemical agents. Unless otherwise stated, all chemicals used were produced by Sigma Chemical Co. (St. Louis, USA).

Semen collection and freezing processing. All animal procedures were approved by the institutional Animal Care and Use Committee of the Domestic Animal Improving Station of Shanxi Province. Semen samples were collected randomly from 5 sexually mature and healthy Qinchuan bulls (aged between 4 and 5 years) from the Domestic Animal Improving Station (Shaanxi Province, China) by artificial vagina. Three samples per bull were collected. Semen samples were incubated in a water bath at 35°C until sperm concentration and initial percentage of motile sperm were estimated. Sperm concentration was evaluated by the optical density using a calibrated spectrophotometer (Shanghai Spectrophotometer Co., Ltd., Shanghai, China). The percentage of motile sperm was estimated at 37°C by phase-contrast microscope at 400× magnification. Only the ejaculates with > 3×10^8 spermatozoa/ml concentration, > 70% motility, and > 85% normal sperm morphology were selected and used for this study. To increase semen volume and eliminate variability between the evaluated samples, the ejaculates were pooled in this study.

Semen processing. After evaluating the quality of the collected semen samples, fresh semen was diluted with cryomidia (2.42 g of Tris, 1.48 g of citric acid, 1.00 g of fructose, 6.6 ml of glycerol, 20 ml of egg yolk, 25 mg of gentamicin, and 50 000 IU of penicillin for 100 ml of deionized water), and poured into polyvinyl chloride (PVC) straws (0.25 ml) (Biovet, Lyon, France). Then the straws were cooled from 37 to 4°C for 1.5 h and kept at 4°C for 2.5 h. The sperm quality was also evaluated after equilibration. Subsequently, the straws were cooled from +4 to –120°C at the speed of 15°C/min (8 min in total). Finally, these straws were transferred to a liquid nitrogen tank (–196°C) for storage.

Semen thawing. Frozen semen samples in the 0.25 ml PVC straws were thawed in a water bath at 37°C for 40 s. All samples were thawed simultaneously at the same time for assessment of sperm motility and total RNA extraction.

Analysis of sperm motility. The percentage of linear motile sperm was observed and evaluated visually by the same technician. For each treatment, three straws of semen were thawed, 10 µl aliquots were put onto glass slides and covered with a glass cover slider and estimated at 37°C by phase-contrast microscope equipped with a warm stage at 400× magnification. Sperm motility was assumed by evaluating the percentage of sperm showing flagellum movement. Sperm motility estimations were performed in 3 different microscopic fields. At least 300 spermatozoa were observed per slide. The mean of 3 successive estimates was recorded as the final sperm motility score and expressed as percentage.

Analysis of membrane integrity. The membrane integrity of the spermatozoa at the three stages was evaluated by using the hypotonic swelling test (HOST) (Osinowo et al. 1982). Briefly, the assay was performed by mixing 50 µl of the semen with 1 ml of hypo-osmotic solution (7.35 g of sodium citrate and 1.351 g of fructose in 1 l of distilled water). After incubation for 60 min at 37°C, sperm swelling was assessed by placing 15 µl well-mixed samples on a warm slide (37°C) under phase-contrast microscopy at 400× magnification and viable spermatozoa had coiled tails after HOST. At least 300 spermatozoa per slide were observed.

RNA extraction and synthesis of cDNA. Total RNA was extracted from semen samples at three different stages (fresh, after equilibration, and frozen-thawed) using improved hot TRIZOL method.

Each semen sample was put into an RNase-free centrifuge tube. After centrifugation (12 000 *g*, 15 min, 4°C), sperm pellets were collected and washed three times in pro-cooled Phosphate Buffered Saline (PBS). The pellet was lysed in 800 µl of TRIZOL Reagent (Invitrogen, Carlsbad, USA) by vortex machine and incubated at room temperature for 5 min. Then, 40 µl dithiothreitol (DTT) were added to the sperm samples. The samples were mixed vigorously and then incubated in a water bath at 65°C for 30 min. The homogenized sample was incubated in an ice bath for 1 min, and then

doi: 10.17221/8239-CJAS

0.2 ml chloroform per 1 ml TRIZOL reagent was added to the sample. Subsequently, the sample was mixed vigorously and then centrifuged (12 000 *g*, 15 min, 4°C). Centrifugation separated the biphasic mixtures into the upper colourless aqueous phase and the lower red phenol-chloroform phase. The RNA was precipitated from the aqueous phase by mixing with 0.5 ml isopropanol (per initial 1 ml TRIZOL reagent). The semen samples were incubated at room temperature for 10 min and centrifuged (12 000 *g*, 10 min, 4°C). The supernatant was removed and the RNA pellet was washed in 1 ml Rnase-free 75% ethanol. The pellet was air dried and dissolved in 30 µl diethyl pyrocarbonate (DEPC)-treated water. Any residual DNA was removed by treating the isolated total RNA with RNase-free DNase 1.

The concentration and quality of total RNA were measured using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, USA) at OD260 and OD280 nm, stored at −80°C until further use. For cDNA synthesis, 1.0 µg total RNA in a final volume of 20 µl was reverse transcribed by PrimeScript RT Reagent Kit (TaKaRa Bio Inc., Otzu, Japan) according to the manufacturer's protocols.

Oligonucleotide primers and PCR amplification. Primers for *HSP70* and *β-actin* used in this study were designed using Premier Primer software (Version 5.0, 2000) based on the mRNA sequences of *β-actin* (GenBank Accession No. AY141970.1) and *HSP70-2* (GenBank Accession No. NM_174344.1) found in Gene Bank. *β-actin* was amplified as an internal control. All primers are shown in Table 1.

The system for PCR is a total volume of 25 µl containing TaKaRa 10X Taq PCR Buffer, $MgCl_2$, dNTP Mixture, the first strand cDNA reaction mixture, forward and reverse specific primers. The PCR was performed under the following conditions: 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 60°C, 30 s at 72°C, and then 10 min at 72°C. PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide.

Quantitative real-time PCR (qRT-PCR) analysis. qRT-PCR was performed using SYBR® Premix Ex Taq II (Tli RNaseH Plus). A total volume of 10 µl contained 6 µl SYBR® Green I Premix, 0.5 µl each of forward and reverse primer, 1.0 µl of cDNA and 2 µl ddH_2O. The thermal cycling profile was the following: one denaturing cycle for 3 min at 95°C, and 40 cycles of PCR (95°C for 10 s, 57°C for 30 s, and 72°C for 30 s). Three replicates were carried out for quantification of the target gene. *β-actin* was amplified for each sample as a house keeping gene for normalization.

Statistical analysis. Data analysis was performed using Statistical Product and Service Solutions (SPSS 13.0 for MS Windows). All values were expressed as mean ± standard error. Analysis of variance (ANOVA) with Tukey's test was used for comparison of mean values at a significance level of $P < 0.05$. For all qPCR experiments, analysis of resultant crossing cycle thresholds (C_T) was performed using the relative comparative C_T method, commonly designated as $2^{-\Delta\Delta C_T}$. The correlation among the above-mentioned methods was evaluated by linear regression analysis.

RESULTS

Sperm motility. Sperm motility at the three stages is shown in Figure 1A. The results showed that the sperm motility in the fresh semen was higher than that of the sperm after equilibration and freezing-thawing ($P < 0.05$), and the sperm motility in the sperm after equilibration was higher than that of the frozen-thawed sperm ($P < 0.05$).

Membrane integrity. Membrane integrity at the three stages is shown in Figure 1B. The results showed that the membrane integrity in the fresh semen was higher than that of the sperm after equilibration and freezing-thawing ($P < 0.05$), and the membrane integrity in the sperm after equilibration was higher than that of the frozen-thawed sperm ($P < 0.05$).

Table 1. Genes and primer sequences

Genes		Primer sequences	Amplification length (bp)	T_a (°C)	GenBank Accession No.
β-actin	forward	5'-ACCCAGCACAATGAAGATCAA-3'	202	60	AY141970.1
	reverse	5'-AACAGTCCGCCTAGAAGCATT-3'			
HSP70-2	forward	5'-TTGGGGACAAGTCAGAGAATG-3'	118	53.9	NM_174344.1
	reverse	5'-ATCGTGGTGTTCCTTTTGATG-3'			

doi: 10.17221/8239-CJAS

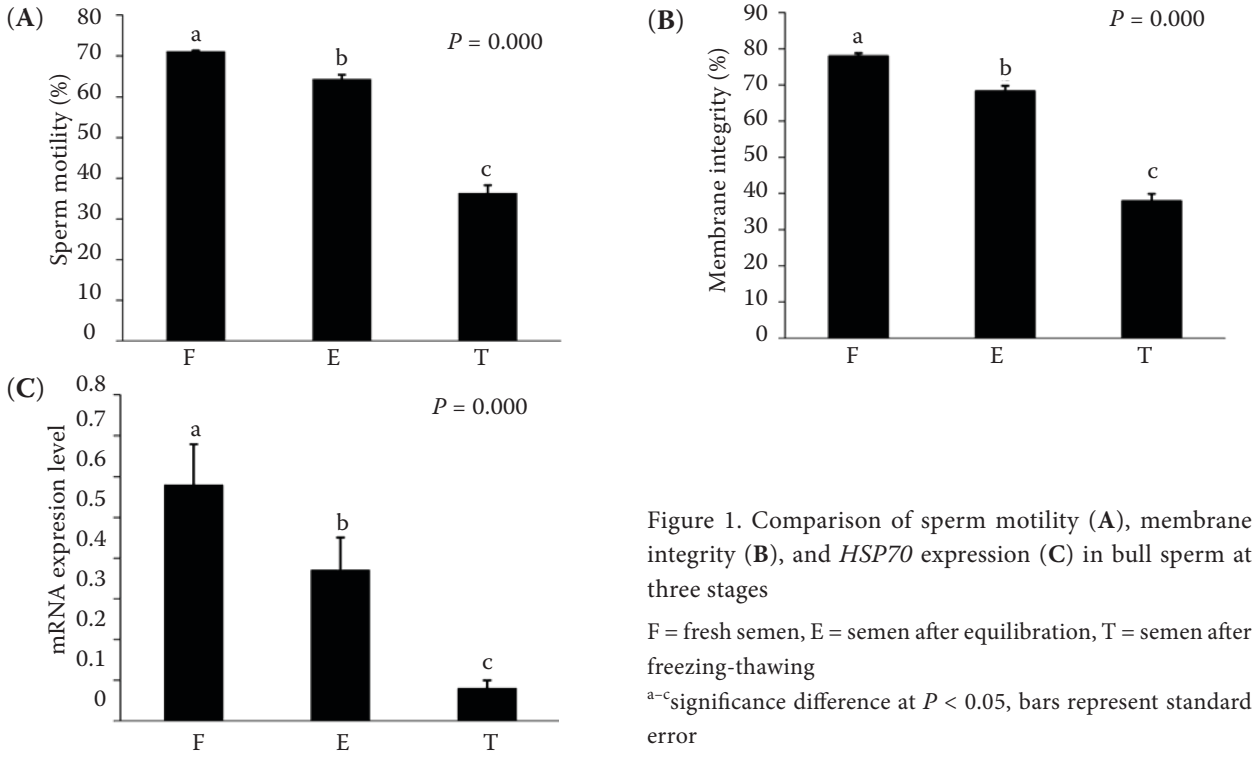

Figure 1. Comparison of sperm motility (**A**), membrane integrity (**B**), and *HSP70* expression (**C**) in bull sperm at three stages

F = fresh semen, E = semen after equilibration, T = semen after freezing-thawing

[a–c]significance difference at $P < 0.05$, bars represent standard error

HSP70 expression level in bull sperm. The *HSP70* expression level at the three stages is shown in Figure 1C. The *HSP70* expression level in bull sperm gradually decreased following the process of freezing-thawing. The *HSP70* expression level in fresh bull sperm was significantly higher than that in the sperm after equilibration ($P < 0.05$). It was also significantly higher in the sperm after equilibration than that in frozen-thawed sperm ($P < 0.05$).

Correlation between HSP70 expression and motility of bull semen at the three stages. Correlation coefficients between *HSP70* expression level and sperm motility at the three stages are summarized in Table 2. The correlation between *HSP70* expression level and sperm motility at the three stages was positive (ranging from 0.327 to 0.785).

DISCUSSION

Recent studies demonstrated that the change of temperature could influence bull sperm quality significantly during cryopreservation (Hu et al. 2011). In this study, in which bull samples were frozen and thawed, the results suggested that motility and membrane integrity of bull sperm gradually decreased following the process of freezing-thawing. The decline in sperm quality caused by the change of temperature was consistent with the previous researches that the decreased temperature resulted in cold shock of sperm and the decline of sperm motility (Roca et al. 2006; Gutierrez-Perez et al. 2009).

HSPs played an important role in resisting stress and maintaining normal cell life activities. The HSPs expression level influenced the acquisition of tolerance in eucaryotic cells (Borkovich et al. 1989). Many studies reported that the HSPs expression level in cells changed when the cell was stimulated by thermal stress and cold stress (Li 1987; Wang et al. 2012). Results in this study were consistent with the above previous studies. In this study, the results suggested that the *HSP70* expression level decreased following the process of freezing-

Table 2. Correlation between *HSP70* expression and sperm motility in bulls' semen samples at three stages (F = fresh semen, E = semen after equilibration, T = semen after freezing-thawing)

Item	F-motility	E-motility	T-motility
F-expression	0.630	–	–
E-expression	0.172	0.785	–
T-expression	0.640	0.240	0.327

doi: 10.17221/8239-CJAS

thawing. Meanwhile, the *HSP70* expression level at the three stages is proportional to sperm motility. Besides the protective function against stresses, HSPs were shown as one of important key factors for sperm fertilizing ability (Casas et al. 2010). A study evaluated *HSP70-2* expression level in ejaculated spermatozoa from 10 oligozoospermic men and 10 normozoospermic men, the results showed a lower expression level in the sperm of infertile men (Lima et al. 2004). These observations together with the results of the present study suggest that motility was a key factor to predicate the fertilizing ability of sperm, and it also indicated that the *HSPs* expression level could affect sperm motility.

Although HSP70 has been regarded as a cytosolic protein, its exact mechanism on sperm function remains unclear. HSPs could prevent the aggregate from misfolded proteins triggered by many types of stresses (Wigley et al. 1999). HSP70 chaperone system could assist non-native folding inter-mediates to fold to the native state (Mayer and Bukau 2005). The decrease of *HSP70* expression level found in this study is likely a degeneration response or chaperonin consumption to maintain protein conformation during the process of freezing-thawing. Results in this study also indicate that the membrane integrity decreased following the process of freezing-thawing, it might be induced by the decrease of *HSP70* expression level. HSP70 interacted with lipids and it had been hypothesized to possibly play a role in the folding of membrane proteins and in the translocation of polypeptides across the membrane (Arispe et al. 2002). It was deduced that synthesizing of HSP70 protein decreased which was caused by the decline of *HSP70* expression level. It resulted in abnormal folding of the proteins on the sperm membrane. The fluidity of sperm membrane might be affected. Aboagla and Terada (2003) indicated that the membrane protein played an important role in the fluidity of sperm membrane. It indicated that sperm motility was decreased due to the decline of sperm membrane fluidity. In the present study, sperm motility, membrane ingerity, and *HSP70* expression level were decreased following the porcess of freezing-thawing ($P < 0.05$). These findings suggested that the decreasing sperm motility might be due to the decreasing *HSP70* expression level. However, the effects of the *HSP70* expression decline on bull sperm motility during the process of freezing-thawing need more studies.

The decline of sperm quality was possibly due to the damage of sperm membrane during the freezing-thawing process. It was well known that the freezing-thawing process could induce physical and chemical stress in sperm plasma membrane that was associated with oxidative stress, and the formation of excess free radical oxygen species (ROS) could lead to impairment of sperm (Hu et al. 2011). HSP70 could regulate cell function by regulating the activity of some enzymes in cells. In our study, sperm motility was decreased while the *HSP70* expression level decreased. It indicated that the change of *HSP70* expression level led to the decline of antioxidant enzymes activities in the cells, relatively more ROS injured sperm, it caused sperm motility decrease. Stressful conditions cause activation of a family of homologous stress-activated proteins, kinases including, and enough HSP70 could inactivate the activity of this protein kinase to restrain cell apoptosis (Gabai et al. 1997). The increasing *HSP70* expression level in cells restrained the activity of protein kinase including p38 and Jun N-terminal Kinase (JNK), which strengthened the cell resistance (Chen and Xu 1998). In our study, *HSP70* expression level and sperm motility showed a positive correlation (ranging from 0.327 to 0.785). Results in this study and previous study suggested that sperm cells resistance to cryopreservation might be strengthened by the increased synthesis of HSP70. HSP70 could activate the activity of Ca^{2+}-ATPase which was inhibited by stress reaction, reduce membrane damage, and relieve the effect of stress reaction on mitochondria (Mestril and Dillmann 1995). Meanwhile, HSP70 could increase superoxide dismutase (SOD) activity when cells underwent heat stress, which protected the cells from oxidative damage. It suggested that HSP70 could protect sperm membrane from ROS by regulating SOD activity. Eukaryotic initiation factor 2α (eIF-2α) is activated in stressed cells which regulate protein synthesis. It mediates both a transient decrease in global translation and the translational up-regulation of selected stress-induced mRNAs (Harding et al. 2000). A previous study indicated that cells would increase the HSPs expression level under stress condition (Guo and Xu 1997). It indicated that when cells are shocked by stress, eIF-2α dephosphorylates to start other proteins synthesis to maintain normal function of cells. However, the results showed that the *HSP70* expression level was decreased

doi: 10.17221/8239-CJAS

following the process of freezing-thawing. It might be the reason that the function of sperm cells was injured beyond their resilience. HSPs was shown to possess an inherent ATPase which was essential for the activation of authentic client proteins *in vivo* (Pearl and Prodromou 2000). Another study indicated that ATP hydrolysis was essential for the chaperone activity of HSP70 proteins (Mayer and Bukau 2005). A direct and positive correlation was found between spermatozoa motility and mitochondrial respiratory complex activities (Eduardo et al. 1998). While sperm had not enough energy to maintain vitality, sperm motility could be declined. Moreover, ATPase could keep its activity only at appropriate temperature, and cold shock reduced ATPase activity. Thus the decreased ATPase activity induced the decline of sperm motility. Our study showed that the decreased temperature led to the decline of sperm motility and the *HSP70* expression level. The correlation between *HSP70* expression and sperm motility at the three stages was positive (ranging from 0.327 to 0.785) which suggested that *HSP70* was expressed at a higher level in sperm with higher motility. The mechanism might be that the increase of *HSP70* level expression restrained ATP degradation, thus sperm had sufficient energy to express more *HSP70* and maintain motility after freezing-thawing.

CONCLUSION

This study has demonstrated that *HSP70* expression level and motility of bull sperm were significantly decreased following the process of freezing-thawing. In addition, the *HSP70* expression level in spermatozoa appears to be correlated with sperm motility in bulls. Nevertheless, further investigations are needed to obtain more information about the exact mechanism of the protective role of HSP70 on sperm and to better understand the exact relationship between *HSP70* expression level and motility of frozen-thawed bull sperm.

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Received: 2014–10–16
Accepted after corrections: 2015–04–02

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