

## HSP90 expression correlation with the freezing resistance of bull sperm

Peng Wang<sup>2</sup>, Yan-Feng Wang<sup>2</sup>, Hong Wang<sup>2</sup>, Chun-Wei Wang<sup>2</sup>, Lin-Sen Zan<sup>2</sup>, Jian-Hong Hu<sup>1</sup>, Qing-Wang Li<sup>1</sup>, Yong-Hong Jia<sup>3</sup> and Guo-Ji Ma<sup>3</sup>

Date submitted: 14.03.2012. Date accepted: 07.06.2012

### Summary

To date, there has been little improvement in cryopreservation of bull sperm due to lack of understanding of the freezing mechanisms. Therefore, this study set out to investigate expression levels of fertility-associated proteins in bull sperm, and in particular the relationship between the 90 kDa heat-shock protein (HSP90) and the sperm characteristics after freezing–thawing. Semen was collected from eight Holstein bulls by artificial vagina. Characteristics of these fresh semen, including sperm motility, morphology, viability and concentration, were evaluated. Sperm quality was also assessed after freezing–thawing. Eight ejaculates were divided into two groups based on freezing resistance and sperm motility. Sperm proteins were extracted and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and western blotting were performed. SDS-PAGE results showed that there was substantial diversity in 90 kDa proteins in the frozen–thawed sperm and HSP90 was confirmed as one of the 90 kDa proteins by western blot. This study indicated that HSP90 expression correlated positively with sperm quality. The amount of expressed 90 kDa proteins in the high freezing resistance (HFR) group was significantly higher than that in the low freezing resistance (LFR) group ( $P < 0.05$ ). Thus, higher expression of HSP90 could probably lead to the higher motility and freezing resistance of sperm found after freezing–thawing. Therefore, we concluded that level of HSP90 expression could be used to predict reliably and simply the freezing resistance of bull sperm.

Keywords: Bull, Freezing resistance, HSP90 expression, Sperm characteristics

### Introduction

Artificial insemination (AI) is currently widely used in cattle-breeding, and the use of frozen–thawed bull semen is a convenient option for this procedure. However, cryopreservation techniques currently in use still result in a loss of 40–50% viable sperm during the freezing–thawing process (Hu *et al.*, 2011). Several parameters can be used to evaluate semen quality after freezing–thawing, such as sperm

motility, sperm survival time and activity of sperm antioxidant enzymes. These parameters could not only forecast the degree of sperm fertility, but also could reflect freezing resistance in diverse individual sperm samples (Casas *et al.*, 2009). The range of sperm proteins includes alkaline proteins, acid proteins and membrane proteins. These proteins participate in energy production in mitochondria (Ruiz-Pesini *et al.*, 1998), the release of nitric oxide (Lewis *et al.*, 1996), protein phosphorylation (Vijayaraghavan *et al.*, 1996), and are also associated with sperm motility after freezing. Recently, several studies have revealed that the semen freezing process, even storage in liquid nitrogen, could cause some damage to the function of sperm proteins (Desrosiers *et al.*, 2006), and to some large molecules such the main proteins in sperm (Cao *et al.*, 2003; Martin *et al.*, 2007). Comparative proteomic analysis of high-fertility and low-fertility bull sperm has shown that several proteins are related to sperm fertility (Gaviraghi *et al.*, 2010), therefore proteomic

<sup>1</sup>Corresponding author: Jian-Hong Hu or Qing-Wang Li. College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi 712100, P.R. China. Tel: +86 29 87092102. Fax: +86 29 87092164. e-mail address: hjh19732008@126.com or liqingwangysu@yahoo.com.cn

<sup>2</sup>College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi 712100, P.R. China.

<sup>3</sup>Domestic Animal Improving Station, Jingyang, Shaanxi 713702, P.R. China.

analysis of sperm could be a valuable tool to identify expression variation of proteins related to freezing resistance and sperm fertility.

Mammalian cells can respond to environmental stress by synthesis of some conserved proteins, such as the family known as heat-shock proteins (HSPs). Among the heat-shock proteins, HSP90 is associated with activation of steroid hormone receptors (Pratt *et al.*, 2004). HSP90 has been found localized in the sperm tail in all species examined and plays a role in mediating sperm fertility. Genetic analysis of viable HSP90 alleles revealed that HSP90 plays a critical role in *Drosophila* spermatogenesis (Yue *et al.*, 1999) and decreased expression of HSP90 might be associated with low sperm motility during cooling of boar sperm (Huang *et al.*, 1999). To further study the novel function of HSP90 on the sperm motility, an HSP90-specific inhibitor geldanamycin (GA) was used and results showed that HSP90 might play a crucial role in regulating boar sperm motility (Huang *et al.*, 2000). In human spermatozoa, the expression level of HSP90 was decreased dramatically after cryopreservation (Cao *et al.*, 2003). During mouse sperm capacitation, HSP90 undergoes tyrosine phosphorylation (Ecroyd *et al.*, 2003). In the field of the bull sperm cryopreservation, only a few studies have been performed to investigate the expression of different protein (such as HSP90) in sperm of different quality.

Therefore, the present study was performed to investigate protein expression in bull sperm of different quality after freezing–thawing by analysis of the protein profile through SDS-polyacrylamide gel electrophoresis (SDS-PAGE). In addition, western blot was used to verify the relationship between HSP90 and frozen–thawed bull sperm quality.

## Materials and methods

### Semen collection and freezing processing

Semen was collected by artificial vagina from eight sexually mature Holstein bulls (4–5 years old) that belonged to the Domestic Animal Improvement Station (Jingyang, Shanxi, China). Semen was incubated in a water bath at 35 °C until the sperm concentration and initial percentage of motile sperm was estimated. Sperm concentration was evaluated by optical density using a calibration spectrophotometer (Shanghai Spectrophotometer Co., Ltd., Shanghai, China). The percentage of motile sperm was estimated at 37 °C by light microscope at  $\times 200$  magnification.

After evaluation of sperm quality, the fresh semen was diluted with commercial refrigeration extender and filled into polyvinyl chloride (PVC) straws

(0.25 ml) (Biovet, Lyon, France). Then the straws were cooled from 37 °C to 4 °C for 1.5 h and kept at 4 °C for 2.5 h. Subsequently, straws were cooled from +4 °C to –120 °C at the speed of approximately 15 °C/min. Finally, these straws were transferred to a liquid nitrogen tank (–196 °C).

### Semen thawing

Semen frozen in the 0.25 ml PVC straws was thawed at 37 °C for 40 s. For assessment of sperm parameters and protein expression, all samples were thawed simultaneously.

### Analysis of the sperm quality

The percentage of linear motile sperm was observed visually. For each bull, four straws of semen were thawed and these samples were transferred into pre-warmed tubes immediately after thawing. After incubation at 37 °C for 5 min, 10- $\mu$ l aliquots were transferred onto glass slides and covered with a glass cover slider. Sperm motility was assessed by evaluating the percentage of sperm that showed flagellum movement. The percentage of motile sperm was estimated at 37 °C by light microscope at  $\times 400$  magnification. At least 300 sperms were counted per slide.

Plasma membrane integrity was evaluated by the hypotonic swelling test (HOST) (Osinowo *et al.*, 1982). Fifty microliters of semen were diluted in 1 ml hypo-osmotic solution that contained 7.35 g sodium citrate and 13.51 g fructose in 1000 ml distilled water. After incubation for 60 min at 37 °C, sperm swelling was assessed by dropping 15  $\mu$ l of well mixed sample on a warm slide (37 °C) and observation under a light microscopy at  $\times 400$  magnification. Viable sperm had coiled tails after HOST. At least 300 sperms per slide were observed. Spermatozoa were classified as positive or negative based on the presence or absence of the coiled tail.

Acrosomal status was evaluated using fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) according to the delineation by Aboagla & Terada (2003). Thirty microliters of the sperm sample were smeared on the microscope slide. Smears were fixed with methanol at 20–22 °C for 10 min and then air-dried. About 30  $\mu$ l FITC-PNA solution (100  $\mu$ g/ml, Sigma) in 1 $\times$  phosphate-buffered saline (PBS, pH 7.4) were spread over each slide. Subsequently, the slides were incubated in a dark and moist chamber for 30 min at 37 °C. After incubation, the slides were rinsed with 1 $\times$  PBS, air-dried and mounted with 10  $\mu$ l of antifade solution to preserve fluorescence. The slide smear was covered by a coverslip and sealed with colorless nail polish. The smears were observed

and photographed by microscope (LEIKA DM-IRB linked up to a Nikon digital camera DXM). The whole acrosome was visualized with strong green fluorescence under a fluorescence microscope and was scored as acrosome-intact sperm. The percentage of fluorescent acrosome-intact sperm was counted in at least 300 sperm cells per slide.

### Experimental design

Semen from eight Holstein bulls was frozen using the freezing process above. After freezing–thawing, sperm quality was assessed. For sperm motility, semen samples were divided into two groups: HFR group in which the sperm motility was >40% after freezing–thawing and LFR group in which the sperm motility was <40% after freezing–thawing. Each group contained four different individual sperm samples. Eight ejaculates, belonging to the HFR or LFR group, were selected to perform the SDS-PAGE and western blot assay. The experiments were repeated three times.

### Preparation of the sperm proteins

After thawing, semen samples were incubated at 37 °C for 1 min, and then were centrifuged (1000 *g*, 4 °C, 5 min) and washed three times in 1 ml of pre-cooled 1 × PBS. The pellet was re-suspended with lysis buffer and incubated at 4 °C for 30 min to extract sperm proteins. After centrifugation (12,000 *g*, 15 min, 4 °C), the supernatant was boiled in the SDS-sample buffer for 5 min. Then the protein concentration was measured on a micro-titer plate using a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Finally, the sperm proteins were stored at –70 °C until use.

### SDS-PAGE

SDS-PAGE was performed to determine the molecular weight and relative content of various sperm proteins. Equal amounts of total proteins from each semen sample were separated by electrophoresis on a 10% SDS-PAGE gel. Pre-stained protein marker (Sigma) was used as a molecular weight standard.

### Western blot

After the sperm proteins were separated on a 10% SDS-PAGE gel, the proteins were transferred to a nitrocellulose (NC) membrane (Bio-Rad, Hercules, CA, USA) using a semi-dry blotting apparatus (Model SD transfer cell, Bio-Rad) at 18 V per mini-gel for 1 h in the transfer buffer. The membrane was subsequently blocked for 30 min in blocking buffer (Tris-buffered saline (TBS) with 3% (w/v) milk and 0.1% (v/v) Tween 20) and then incubated at 4 °C overnight

with the goat monoclonal anti-HSP90 antibody (Santa Cruz Biotechnology, Inc. California, USA), which was diluted to 1:1000 (v:v) in the blocking buffer. Then the membrane was washed three times with 0.1% TBST and incubated with the rabbit conjugate anti-goat IgG–horseradish peroxidase (HRP) (1:5000 diluted in the blocking buffer) for 1 h at room temperature. Finally, the membrane was washed three times with 0.1% TBST, and developed for 5 min with the chemiluminescent HRP substrate (Immobilized Western Detection System, Millipore Corporation, Billerica, MA, USA).

### Image acquisition and data analysis

Gel images and the protein patterns were scanned and quantified using a gel documentation system (Gel Doc XR; Bio-Rad) and Quantity One Version 4.6.5 software package (Bio-Rad). The protein levels were expressed by 'band volume', which was defined as the total signal intensity inside the boundary of a band measured in pixel intensity units (density) × mm<sup>2</sup>, the lowest intensity in a pixel is zero (white). To avoid background signals, band boundaries were defined on amplified digital images of the protein patterns. β-Actin was used as an internal standard to normalize the volume of protein bands.

Data analysis was performed using the SPSS software program (version 13.0). All values were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) with Tukey's test was used for comparison of mean values at a significance level of  $P < 0.05$ .

## Results

### Post-thaw viability

Frozen–thawed bull sperm motility, membrane integrity and acrosome integrity are shown in Table 1. The results showed that sperm quality from different bulls varied markedly. Eight ejaculates were classified into two clusters (HFR and LFR group, Table 2) based on the maximal dissimilarities of the sperm progressive motility after freezing–thawing. Ejaculate samples nos. 1, 2, 3 and 6 were assigned to the LFR group in which the sperm progressive motility was lower than 40% after freezing–thawing. Ejaculate semen samples nos. 5, 7, 8 and 9 were assigned to the HFR group in which sperm progressive motility exceeded 40% after freezing–thawing. Sperm quality parameters in the HFR group were significantly higher than those in the LFR group ( $P < 0.05$ ). These eight ejaculates semen samples were used to perform western blot.

**Table 1.** Motility, membrane integrity and acrosome integrity of bull sperm after freezing–thawing

Semen samples	Sperm motility (%)	Membrane integrity (%)	Acrosome integrity (%)
1	35.1 ± 0.2 <sup>d</sup>	37.3 ± 0.9 <sup>e</sup>	33.7 ± 4.1 <sup>e</sup>
2	39.8 ± 0.1 <sup>c</sup>	41.3 ± 2.1 <sup>d,e</sup>	40.3 ± 2.1 <sup>c,d</sup>
3	36.3 ± 0.4 <sup>d</sup>	36.7 ± 2.0 <sup>e</sup>	37.2 ± 2.1 <sup>d,e</sup>
4	41.5 ± 0.4 <sup>c</sup>	45.4 ± 1.9 <sup>c,d</sup>	42.5 ± 4.9 <sup>b,c,d</sup>
5	44.3 ± 0.1 <sup>b</sup>	51.8 ± 2.1 <sup>b</sup>	45.8 ± 1.9 <sup>a,b,c</sup>
6	39.6 ± 0.6 <sup>c</sup>	40.2 ± 5.1 <sup>d,e</sup>	40.1 ± 5.9 <sup>c,d</sup>
7	43.7 ± 2.0 <sup>b</sup>	50.2 ± 3.2 <sup>b,c</sup>	43.6 ± 1.9 <sup>b,c,d</sup>
8	52.7 ± 2.1 <sup>a</sup>	63.1 ± 4.1 <sup>a</sup>	51.1 ± 3.9 <sup>a</sup>
9	45.2 ± 2.1 <sup>b</sup>	52.5 ± 3.0 <sup>b</sup>	48.2 ± 1.1 <sup>a,b</sup>

Note: Different superscript letters (a–e) in the same column represents significance difference at  $P < 0.05$ .

**Table 2.** Classification of ejaculate semen samples

Sperm quality parameters	Clusters	
	HFR group	LFR group
Ejaculate number	5, 7, 8, 9	1, 2, 3, 6
Sperm progressive motility (%)	46.47 ± 4.19 <sup>a</sup>	37.70 ± 2.36 <sup>b</sup>
Sperm membrane integrity (%)	54.40 ± 5.87 <sup>a</sup>	38.87 ± 2.22 <sup>b</sup>
Sperm acrosome integrity (%)	47.17 ± 3.22 <sup>a</sup>	37.82 ± 3.09 <sup>b</sup>

Note: Values in the same row marked with different letters means significantly difference at  $P < 0.05$ . HFR: high freezing resistance; LFR: low freezing resistance.

### Sperm proteins expressed in the HFR and LFR groups

Thirteen prominent protein bands were observed in which molecular weights ranged from 2.8–147.4 kDa. The band volumes of the 90 kDa protein in the HFR group were significantly higher than those in the LFR group ( $P < 0.05$ ).

### HSP90 expression level in the HFR and LFR groups

The most representative protein patterns are shown in Figs. 1 and 2. The average expression level of HSP90

**Table 3.** Correlation between HSP90 expression and different parameters for evaluating quality of bull sperm after freezing–thawing

Item	HSP90 expression	Sperm motility	Membrane integrity	Acrosome integrity
HSP90 expression	–			
Sperm motility	0.364	–		
Membrane integrity	0.447	0.985	–	
Acrosome integrity	0.402	0.965	0.953	–

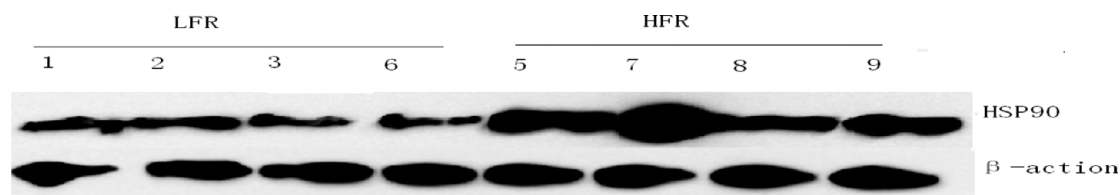
in the LFR group was significantly lower than that in the HFR group ( $P < 0.05$ ). Also, the relative expression level of HSP90 among different ejaculates in the LFR group were similar, however, those in the HFR group were markedly different. HSP90 proteins were especially highly expressed in nos. 5 and 7 ejaculates compared with those of nos. 8 and 9. In general, the more sensitive the bull sperm to cold shock, the lower the expression of HSP90 after freezing–thawing. HSP90 was expressed more highly in sperm with higher motility and vice versa.

### HSP90 expression was moderately related to the sperm quality parameters

Correlation coefficients among different tests (HSP90 expression, sperm motility, acrosome integrity and membrane integrity) are summarized in Table 3. The correlation between HSP90 expression and sperm quality parameters was of moderate strength (ranging from 0.364–0.447).

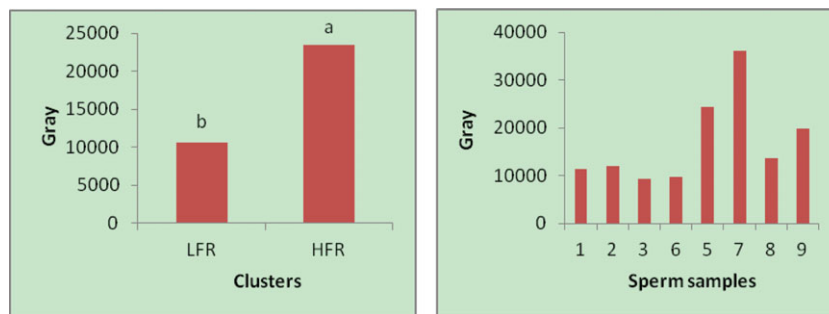
## Discussion

In this study, in which bull sperm was frozen and thawed, a change in quality parameters for bull sperm, including progressive motility, membrane integrity and acrosome integrity, was found. The degree of



**Figure 1.** Expression pattern of 90 kDa heat-shock proteins (HSP90) in high freezing resistance (HFR) and low freezing resistance (LFR) sperm detected using western blot.





**Figure 2.** (Colour online) Expression of 90 kDa heat-shock proteins (HSP90). Note: The Gray value indicates expression of sperm HSP90 in clusters from different individual bulls. The unit is integral optical density (IOD). Different superscript letters (a, b) indicates significance difference at  $P < 0.05$ .

variation caused by cold shock was different in sperm in different ejaculates, including those that had undergone the same cryopreservation conditions. These results were consistent with results from previous studies (Roca *et al.*, 2006; Gutierrez-Perez *et al.*, 2009).

Previously, 125 putative biomarkers of fertility have been identified when high- and low-fertility bull sperm were evaluated by differential detergent fractionation multidimensional protein identification technology (DDF-Mud PIT) (Peddinti *et al.*, 2008). Sperm from high-fertility bulls had a higher expression of proteins that were involved in energy metabolism, cell communication, spermatogenesis, and cell motility compared with sperm from low-fertility bulls. HSPs have been indicated as important key factors for the fertilizing ability of sperm in addition to having a protective function against stress (Casas *et al.*, 2010). In a previous study, it was shown that HSP90 levels were markedly different between ejaculates that had either a good or poor freezing ability (Casas *et al.*, 2010). In this study it was found that HSP90 from sperm from the LFR group displayed weaker reactivity compared with that from the HFR group. Therefore, it was possible to predict the potential freezing resistance ability of ejaculates based on the expression level of HSP90 by western blot, as sperm with a higher level of HSP90 had more resistance to freezing and vice versa.

Although HSP90 has been regarded as a cytosolic protein, its exact function still remains unclear. HSP90 plays an essential role in stress tolerance (Borkovich *et al.*, 1989), protein folding (Freeman & Morimoto, 1996), signal transduction (Pratt, 1998), and so on. Many proteins, including tyrosine kinases, serine-threonine kinases and other enzymes, may associate with HSP90 to affect the sperm motility (Pratt, 1998). HSP90 is a ubiquitous molecular chaperone that provides resistance against cell oxidative stress, apoptosis, and mediates cell protection and protein folding during thermal stress (Fukuda *et al.*, 1996; Powers *et al.*, 2008). It has been shown that the

expression of HSP90 in HeLa cells was decreased significantly after cryopreservation (Wang *et al.*, 2005). Also, HSP90 could activate nitric oxide synthase (NOS), which is beneficial to sperm motility within the physiological range (Garcia-Cardena *et al.*, 1998). Nitric oxide (NO) is a reactive nitrogen species synthesized by NOS that may act as an antioxidant or free radical in inter-cellular and intra-cellular signalling (Dixit & Parvizi, 2001). It is well known that the freezing–thawing process could induce physical and chemical stress in the sperm plasma membrane that is associated with oxidative stress and the formation of free radical oxygen species (ROS), and resulting in impairment of sperm (Alvarez & Storey, 1992). It has been found that HSP90 can protect cells from ROS (Fukuda *et al.*, 1996). However, the protective roles of HSP90 against oxidative stress during semen freezing remain to be determined. In view of these studies, we could hypothesize that HSP90 might participate in the regulation of the sperm motility through modulation of the activity of its binding protein kinases. The freezing–thawing process results in cell oxidation and apoptosis in sperm, and HSP90 can protect sperm from this change through direct resistance to ROS or activation of NOS to produce NO that will eliminate ROS. Therefore, HSP90 could protect sperm from oxidative damage and improve sperm quality after freezing–thawing. In addition, HSP90 might contribute to changes during repair that occur in protamine–DNA complexes and in structural damage to chromatin during boar sperm cryopreservation (Flores *et al.*, 2008).

HSP90 had been shown to possess an inherent ATPase that is essential for the activation of authentic client proteins *in vivo* (Pearl & Prodromou, 2000). It has been claimed that the ATP level in sperm cell membranes diminishes after cold shock and is not restored subsequently (Watson, 1981). The diminishing levels of ATP might result in reduction in sperm motility. It has been demonstrated that HSP90 is involved in ATP metabolism (Prodromou *et al.*,

1997). During freezing–thawing, HSP90 initiates ATP depletion, which leads to lack of sperm dynamics such that the sperm stays in a resting state to reduce consumption of energy and reduce damage. Sperm motility functions could be affected either by the decrease in ATP, which is concomitant with lower mitochondrial potential (Pena *et al.*, 2009), or by structural damage in the contractile apparatus of the flagellum (Guthrie *et al.*, 2008). Our study showed that there were positive correlations between HSP90 expression and sperm motility, membrane integrity, and acrosome integrity; the correlation coefficient between HSP90 expression and sperm motility was 0.364. Moreover, HSP90 was expressed at a higher level in sperm with higher motility. A possible mechanism is that a surfeit of HSP90 inhibits ATP degradation and provides sufficient energy to ensure that spermatozoa have higher motility after freezing–thawing. These results illustrate that a higher HSP90 expression accurately predicts the increased freezing resistance of the sperm. Nevertheless, more research needs to be performed to investigate the mechanism of the protective role of HSP90 and to better understand the exact relationship between HSP90 expression and the sperm quality.

## Conclusion

In this study, we found that expression of HSP90 in HFR bull sperm after freezing–thawing was dramatically higher than that in LFR bull sperm and, in addition, higher expression of HSP90 was related to lower sensitivity of HFR sperm to cold shock. Therefore, HSP90 could be considered as a cold shock molecular marker for frozen bull semen. This study indicated the need to search for cold shock markers to improve freezing ability prediction tests for frozen semen ejaculates, an approach that would have a high impact on bull semen cryopreservation methods.

## Acknowledgements

This research was supported by the 12th Five-year Plan Rural Areas State Science and Technology Support Projects (2011BAD19B04-3), National Agriculture Science and Technology Achievements Transfer Capital Support Project (2010GB2G000466), Program for Yangtze Rive Scholar and Innovative Research Team of the Ministry of Education (IRT0940), the '13115' Sci-Tech Innovation Project of Shaanxi Province (2010ZDGC-01), Scientific and Technological Project of Shaanxi Province (2011K01-04 and 2010K01-05), Agricultural Sci-Tech Innovation Project of Shaanxi Province

(2011NXC01-11), Science and Technology Innovation Engineering Project of Shaanxi Province (2011KTCL02-11), the Basic Scientific Research Expense of Major Project of Northwest A&F University (PY200901) and the Basic Scientific Research Expense of Sci-Tech Innovation Major Project of Northwest A&F University (QN2011061).

## References

- Aboagla, E.M.E. & Terada, T. (2003). Trehalose-enhanced fluidity of the goat sperm membrane and its protection during freezing. *Biol. Reprod.* **69**, 1245–50.
- Alvarez, J.G. & Storey, B.T. (1992). Evidence for increased lipid peroxidative damage and loss of superoxide-dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. *J. Androl.* **13**, 232–41.
- Borkovich, K.A., Farrelly, F.W., Finkelstein, D.B., Taulien, J. & Lindquist, S. (1989). Hsp82 is an essential protein that is required in higher concentrations for growth of cells at higher temperatures. *Mol. Cell Biol.* **9**, 3919–30.
- Cao, W.L., Wang, Y.X., Xiang, Z.Q. & Li, Z. (2003). Cryopreservation-induced decrease in heat-shock protein 90 in human spermatozoa and its mechanism. *Asian J. Androl.* **5**, 43–6.
- Casas, I., Sancho, S., Briz, A., Pinart, E., Bussalleu, E., Yeste, M. & Bonet, S. (2009). Freezability prediction of boar ejaculates assessed by functional sperm parameters and sperm proteins. *Theriogenology* **72**, 930–48.
- Casas, I., Sancho, S., Ballester, J., Briz, M., Pinart, E., Bussalleu, E., Yeste, M., Fabrega, A., Rodriguez-Gil, J.E. & Bonet, S. (2010). The HSP90AA1 sperm content and the prediction of the boar ejaculate freezability. *Theriogenology* **74**, 940–50.
- Desrosiers, P., Legare, C., Leclerc, P. & Sullivan, R. (2006). Membranous and structural damage that occur during cryopreservation of human sperm may be time-related events. *Fertil. Steril.* **85**, 1744–52.
- Dixit, V.D. & Parvizi, N. (2001). Nitric oxide and the control of reproduction. *Anim. Reprod. Sci.* **65**, 1–16.
- Ecroyd, H., Jones, R.C. & Aitken, R.J. (2003). Tyrosine phosphorylation of HSP-90 during mammalian sperm capacitation. *Biol. Reprod.* **69**, 1801–7.
- Flores, E., Cifuentes, D., Fernandez-Novell, J.M., Medrano, A., Bonet, S., Briz, M.D., Pinart, E., Pena, A., Rigau, T. & Rodriguez-Gil, J.E. (2008). Freeze-thawing induces alterations in the protamine-1/DNA overall structure in boar sperm. *Theriogenology* **69**, 1083–94.
- Freeman, B.C. & Morimoto, R.I. (1996). The human cytosolic molecular chaperones hsp90, Hsp70 (hsc70) and hdj-1 have distinct roles in recognition of a non-native protein and protein refolding. *EMBO J.* **15**, 2969–79.
- Fukuda, A., Osawa, T., Oda, H., Tanaka, T., Toyokuni, S. & Uchida, K. (1996). Oxidative stress response in iron-induced acute nephrotoxicity: Enhanced expression of heat-shock protein 90. *Biochem. Biophys. Res. Co.* **219**, 76–81.
- Garcia-Cardena, G., Fan, R., Shah, V., Sorrentino, R., Cirino, G., Papapetropoulos, A. & Sessa, W.C. (1998). Dynamic

- activation of endothelial nitric oxide synthase by Hsp90. *Nature* **392**, 821–4.
- Gaviraghi, A., Deriu, F., Soggiu, A., Galli, A., Bonacina, C., Bonizzi, L. & Roncada, P. (2010). Proteomics to investigate fertility in bulls. *Vet. Res. Commun.* **34**, S33–6.
- Guthrie, H.D., Welch, G.R. & Long, J.A. (2008). Mitochondrial function and reactive oxygen species action in relation to boar motility. *Theriogenology* **70**, 1209–15.
- Gutierrez-Perez, O., de Lourdes Juarez-Mosqueda, M., Uribe Carvajal, S. & Trujillo Ortega, M.E. (2009). Boar spermatozoa cryopreservation in low glycerol/trehalose enriched freezing media improves cellular integrity. *Cryobiology* **58**, 287–92.
- Hu, J.H., Zhao, X.L., Tian, W.Q., Zan, L.S. & Li, Q.W. (2011). Effects of vitamin E supplementation in the extender on frozen-thawed bovine semen preservation. *Animal* **5**, 107–12.
- Huang, S.Y., Kuo, Y.H., Lee, W.C., Tsou, H.L., Lee, Y.P., Chang, H.L., Wu, J.J. & Yang, P.C. (1999). Substantial decrease of heat-shock protein 90 precedes the decline of sperm motility during cooling of boar spermatozoa. *Theriogenology* **51**, 1007–16.
- Huang, S.Y., Kuo, Y.H., Tsou, H.L., Lee, Y.P., King, Y.T., Huang, H.C., Yang, P.C. & Lee, W.C. (2000). The decline of porcine sperm motility by geldanamycin, a specific inhibitor of heat-shock protein 90 (HSP90). *Theriogenology* **53**, 1177–84.
- Osinowo, O.A., Bale, J.O., Oyedipe, E.O. & Eduvie, L.O. (1982). Motility and eosin uptake of formaldehyde-treated ram spermatozoa. *J. Reprod. Fertil.* **65**, 389–94.
- Lewis, S.E., Donnelly, E.T., Sterling, E.S., Kennedy, M.S., Thompson, W. & Chakravarthy, U. (1996). Nitric oxide synthase and nitrite production in human spermatozoa: evidence that endogenous nitric oxide is beneficial to sperm motility. *Mol. Hum. Reprod.* **2**, 873–8.
- Martin, G., Cagnon, N., Sabido, O., Sion, B., Grizard, G., Durand, P. & Levy, R. (2007). Kinetics of occurrence of some features of apoptosis during the cryopreservation process of bovine spermatozoa. *Hum. Reprod.* **22**, 380–8.
- Pearl, L.H. & Prodromou, C. (2000). Structure and in vivo function of Hsp90. *Curr. Opin. Struct. Biol.* **10**, 46–51.
- Peddinti, D., Nanduri, B., Kaya, A., Feugang, J.M., Burgess, S.C. & Memili, E. (2008). Comprehensive proteomic analysis of bovine spermatozoa of varying fertility rates and identification of biomarkers associated with fertility. *Bmc. Syst. Biol.* **2**, 19.
- Pena, F.J., Rodriguez Martinez, H., Tapia, J.A., Orteag Ferrusola, C., Gonzalez Fernandez, L. & Macias Garcia, B. (2009). Mitochondria in mammalian sperm physiology and pathology: a review. *Reprod. Domest. Anim.* **44**, 345–9.
- Powers, M.V., Clarke, P.A. & Workman, P. (2008). Dual targeting of HSC70 and HSP72 inhibits HSP90 function and induces tumor-specific apoptosis. *Cancer Cell* **14**, 250–62.
- Pratt, W.B. (1998). The hsp90-based chaperone system: Involvement in signal transduction from a variety of hormone and growth factor receptors. *Proc. Soc. Exp. Biol. Med.* **217**, 420–34.
- Pratt, W.B., Galigniana, M.D., Harrell, J.M. & DeFranco, D.B. (2004). Role of hsp90 and the hsp90-binding immunophilins in signalling protein movement. *Cell Signal.* **16**, 857–72.
- Prodromou, C., Roe, S.M., O'Brien, R., Ladbury, J.E., Piper, P.W. & Pearl, L.H. (1997). Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell* **90**, 65–75.
- Roca, J., Hernandez, M., Carvajal, G., Vazquez, J.M. & Martinez, E.A. (2006). Factors influencing boar sperm cryosurvival. *J. Anim. Sci.* **84**: 2692–9.
- Ruiz-Pesini, E., Diez, C., Lapena, A.C., Perez-Martos, A., Montoya, J., Alvarez, E., Arenas, J. & Lopez-Perez, M.J. (1998). Correlation of sperm motility with mitochondrial enzymatic activities. *Clin. Chem.* **44**, 1616–20.
- Vijayaraghavan, S., Stephens, D.T., Trautman, K., Smith, G.D., Khatra, B., Silva, E. & Greengard, P. (1996). Sperm motility development in the epididymis is associated with decreased glycogen synthase kinase-3 and protein phosphatase 1 activity. *Biol. Reprod.* **54**, 709–18.
- Wang, P.T., Shu, Z.Q., He, L.Q., Cui, X.D., Wang, Y.Z. & Gao, D.Y. (2005). The pertinence of expression of heat-shock proteins (HSPs) to the efficacy of cryopreservation in HELAS. *Cryoletters* **26**, 7–16.
- Watson, P.F. (1981). The effects of cold shock on sperm cell membranes. In: *Effects of Low Temperature on Biological Membranes* (Morris, G.J. & Clarke, A., eds). London: Academic Press, pp. 189–218.
- Yue, L., Karr, T.L., Nathan, D.F., Swift, H., Srinivasan, S. & Lindquist, S. (1999). Genetic analysis of viable Hsp90 alleles reveals a critical role in *Drosophila* spermatogenesis. *Genetics* **151**, 1065–79.