Theriogenology xxx (2012) 1–10

Contents lists available at SciVerse ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

The addition of heat shock protein HSPA8 to cryoprotective media improves the survival of brown bear (*Ursus arctos*) spermatozoa during chilling and after cryopreservation

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ARTICLE INFO

Article history:
Received 21 September 2012
Accepted 1 November 2012

Keywords:
Brown bear
Sperm cryopreservation
Heat shock proteins
Artificial mucus

ABSTRACT

The Cantabrian brown bear survives as a small remnant population in northern Spain and semen cryopreservation for future artificial insemination is one of the measures being implemented for conservation of this species. As part of this program we investigated the value of adding heat shock protein A8 (HSPA8) to media (TES-TRIS-fructose with 20% egg yolk) used for chilling and cryopreserving the spermatozoa. Semen samples from eight brown bears were obtained by electroejaculation during the breeding season. In experiment 1, we tested three concentrations of HSPA8 (0.5, 1, and 5 μg/mL) to determine whether sperm motility (CASA system) and sperm survival could be improved during refrigeration (5 °C) up to 48 hours. Results showed that sperm viability (test with propidium iodide) was improved by the addition of 0.5 and 5 μg/mL HSPA8. In experiment 2, HSPA8 was added to the cryopreservation media (6% final glycerol concentration) before the freezing process. Though there were no differences in sperm viability immediately after thawing (analyses to 0 hours), plasma membrane permeability (test with YO-PRO-1) was significantly lower by the presence of HSPA8 (1 μg/mL) and acrosomal damage (test with PNA-FITC) was reduced by higher concentrations of HSPA8 (1 and 5 μg/mL) (analyses after thermal stress test incubating over 2 hours to 37 °C). In experiment 3, results of a simple progression test carried out through artificial mucus (hyaluronic acid 4 mg/mL) showed a significant decrease in the total number of sperm able to swim a distance of 0.5 to 2 cm through a capillary tube for all HSPA8-based extenders. Nevertheless, the distance traveled by the vanguard spermatozoa, which represent a highly motile subpopulation, was restored by the inclusion of 1 and 5 μg/mL HSPA8 in the cryopreservation media. Thus, the HSPA8 addition to extender improves the quality of brown bear (*Ursus arctos*) sperm during chilling (viability) and after cryopreservation (number of sperm with damaged acrosomes and "apoptotic-like" changes).

1. Introduction

The Cantabrian brown bear (*Ursus arctos*) is the last indigenous brown bear population in the Iberian Peninsula and is considered to be at risk of extinction (Real Decreto 439/1990, regulation of the National Catalogue of Endangered Species) because of habitat loss and population fragmentation (approximately 150 individuals restricted to two isolated populations in the North of Spain). The development of a genome resource bank for this species, mainly consisting of frozen semen for future use with

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http://dx.doi.org/10.1016/j.theriogenology.2012.11.006

artificial insemination, has been proposed as a useful adjunct to other conservation methods because it would maximize the reproductive potential of the male by overcoming the constraints of time and space [1].

The development of a species-specific conservation protocol that includes artificial insemination with frozen semen has to include the validation of an effective method for freezing semen [2]. The application of these technologies must be adapted to individual species because spermatozoa clearly respond differently depending on the origin of the sample, creating a need to conduct research into sperm cryopreservation [3].

In brown bears, recent studies have focused on the optimization of extender composition, including egg yolk [4–6], glycerol [7,8], and several additives [9], and glycerol concentration and freezing rate used [10]. Together, these studies have resulted in improved methods for semen storage *in vitro*; however, recent investigations have indicated that there might be some additional benefits to be gained by exploiting the physiological ability of the female reproductive tract to prolong sperm viability *in vivo*. Several studies of sperm storage in the mammalian female reproductive tract have reported that the ascent of spermatozoa is regulated by the female, and that a successful spermatozoon must be equipped with a particular suite of proteins for entry into the oviduct, and then remain viable until ovulation has occurred [11].

Moreover, it is increasingly believed that the oviduct is not a passive conduit for the circulation of spermatozoa and that a sperm–oviduct dialogue is an important feature of sperm transport [12]. Contact between the spermatozoa and the surface of oviductal epithelial cells has developed a new paradigm in this regard, by showing that the arrival of spermatozoa stimulates the *de novo* expression of genes and the synthesis of novel proteins [13–15]. Heat shock proteins are among the novel proteins studied and experimentally it has been shown that one of these (heat shock protein A8 (HSPA8); formerly known as heat shock cognate protein 73 kD [HSC70]) is able to prolong the viability of boar, bull, and ram fresh spermatozoa *in vitro* [16,17]. Because this specific protein is highly conserved across species (for review, see [18]) and its bioactivity for mammalian spermatozoa is not species-specific, the objective of this study was to determine whether the protein could also be beneficial for the long- (cryopreservation) and short-term (chilling at 5 °C) storage of brown bear spermatozoa. Apart from evaluating spermatozoa by the use of routine parameters such as viability, motility, and acrosomal integrity, we also tested the frozen/thawed spermatozoa for their ability to migrate through capillary tubes containing hyaluronic acid. This method is regarded as providing physiologically-relevant information about the ability of spermatozoa to colonize the oviduct and penetrate oocytes during *in vitro* fertilization [19].

2. Materials and methods

2.1. Reagents and animal regulation

All the products were obtained from Sigma (Madrid, Spain), except Equex STM Paste (Minitüb, Tiefenbach, Germany); bovine recombinant HSPA8 was provided by Dr. Fazeli (The Academic Unit of Reproductive and Developmental Medicine, University of Sheffield, UK).

2.2. Animals and sample collection

Semen samples from eight sexually mature male Brown bears were obtained by electroejaculation during the breeding season (late April to early July). Animals were housed in a half-freedom regime in Cabárceno Park (Cantabria, Spain; 43° 21′ N, 3° 50′ W, altitude: 142 m), and fed with a diet based on chicken meat, bread, and fruit. Animal manipulations were performed in accordance with Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65.

The males were immobilized by teleanesthesia, using 750 mg of zolazepam HCl with tiletamine HCl (Zoletil100; Virbac, Carros, France) and 6 mg of medetomidine (Zalopin, Orion Pharma Animal Health, Finland; 10 mg/mL). After immobilization, the males were weighed and monitored (pulse, oxygen saturation, and respiration). Before electroejaculation, the prepucial area was shaved and washed with physiological saline serum, and the rectum was emptied of feces. The bladder was catheterized during semen collection to prevent urine contamination. Electroejaculation was carried out with a PT Electronics electroejaculator (Boring, OR, USA). The transrectal probe was 320 mm long with a diameter of 26 mm. Electric stimuli were given until ejaculation (10 V and 250 mA, on average).

To prevent urine contamination or low cellular concentration, the ejaculates were collected as isolated fractions in 15 mL graduated glass tubes. Immediately after collection, the volume of each fraction was recorded, osmolality was measured using a cryoscopic osmometer Osmomat-030 (Gonotec; Berlin, Germany) and the pH value was determined by a CG 837 pH meter (Schott Instruments, Main, Germany). For each fraction, subjective motility was assessed with a phase contrast microscope (magnification × 100) and urospermia checked by DiaSys Ecoline test (Diagnostics Systems GmbH, Holzheim, Germany). Fractions with a reduced concentration ($<150 \times 10^6$ cells per mL), low motility (<50%), or contaminated urine (>80 mg urea per dL) were rejected. All valid fractions of the same electroejaculation were mixed and constituted one ejaculate. The selected samples were centrifuged at 600 × *g* for 6 minutes and the pellet was processed according to the experimental design.

2.3. Experimental design

Two extenders were prepared. The control extender (EY) for refrigeration storage was TES-TRIS-fructose 300 mOsm/kg, pH 7.1, with 2% EDTA and 1% Equex STM paste with 20% egg yolk (based on Anel et al. [4], without glycerol). The EY for cryopreservation was the same as for refrigeration process but 6% (vol/vol) glycerol was added (Anel et al. [4], modified by de Paz et al. [10]).

2.3.1. Experiment 1. Use of HSPA8 for the refrigeration storage (5 °C) of semen samples

The pellets were divided into four aliquots and extended 1:1 with four different extenders: EY (0 μg/mL HSPA8), EY

with 0.5 μg/mL HSPA8 (HA), EY with 1 μg/mL HSPA8 (HB), and EY with 5 μg/mL HSPA8 (HC). The tubes with the extended samples were put into beakers containing 100 mL of water at room temperature (22 °C) and transferred to a refrigerator, where samples were slowly cooled (−0.25 °C per minute) to 5 °C. Then, the samples were diluted with the appropriate volume of each extender to achieve the final sperm concentration (100×10^6 sperm per mL). After that, the samples were refrigerated (5 °C) to 48 hours and analyzed for motility and membrane status parameters at 0, 24, and 48 hours (see section 2.4.).

2.3.2. Experiment 2. Use of HSPA8 in the cryopreservation of semen samples

The samples were divided in four aliquots and extended 1:1 with four different extenders: EY, HA, HB, and HC. The tubes with the extended samples (1:1 with 3% glycerol extender [TES-TRIS-fructose 300 mOsm/kg, pH 7.1, with 2% EDTA and 1% Equex STM paste] with 20% egg yolk [4]) were put into beakers containing 100 mL of water at room temperature (approximately 22 °C) and transferred to a refrigerator, where they were slowly cooled (−0.25 °C per minute) to 5 °C. Then, the samples were diluted with the same volume of each extender containing 9% glycerol, to achieve the final glycerol concentration of 6% [10]. Final sperm concentration (100×10^6 spermatozoa per mL) was achieved by adding the appropriate volume of each extender to the 6% glycerol. After packaging into 0.25-mL plastic straws and equilibrating for 1 hour at 5°C, the samples were frozen in a programmable biofreezer (Kryo 10 Series III; Planer Plc., Sunbury-On-Thames, UK) at −20 °C per minute to −100 °C, and then transferred to liquid nitrogen containers. The cryopreserved samples remained in liquid nitrogen for a minimum of 1 week. Thawing was performed by dropping the straws into water at 65 °C for 6 seconds. After that, an aliquot of sperm sample was analyzed (See section 2.4.) and another aliquot was subjected to a thermal stress test, incubating to 37 °C over 2 hours, to check for latent damage to the sperm (adapted from Alvarez-Rodríguez et al. [7]).

2.3.3. Experiment 3. Assessment of frozen-thawed spermatozoa using a hyaluronic acid migration test

The sperm migration test was carried out immediately after thawing the samples. The sperm samples (200 μL [100×10^6 sperm per mL]) were placed in small glass tubes. Then, flat capillary tubes (VITROTUBES capillary [Mod. 3520-050 (0.20 × 2.00 × 50 mm); CM Scientific Ltd., Silsden, UK]) were filled with a solution of hyaluronic acid: 4 mg/mL hyaluronic acid (Sigma-Aldrich; 53747; 1.5 to 1.8 $\times 10^6$ d) in a PBS buffer containing 0.05% BSA (to avoid sperm agglutination). The capillaries were placed inside the glass tube containing the sperm suspension and incubated at 39 °C for 10 minutes. Thereafter, sperm progression was assessed under a phase-contrast microscope (magnification × 200) (Olympus BH2; negative-high phase contrast). The distance in mm traveled by the vanguard spermatozoon (spermatozoa capable of achieving the greatest distance in the capillary) and the number of spermatozoa per capillary tube accumulated between 0.5 and 2 cm from the base of the capillary were measured. The first segment (0 to 0.5 cm) was discarded because of sperm concentration because of direct sample contact, which was not significant to real sperm progression [20]. Sperm counts that progressed beyond 2 cm were irrelevant.

2.4. Semen evaluation

Sperm quality was evaluated at different points: refrigeration storage times (0, 24, and 48 hours) for experiment 1 and before freezing and after thawing (postthawing and after 2-hour incubation [37 °C]) for experiment 2.

2.4.1. Sperm motility

The assessment of motility parameters was performed using a computer assisted semen motility analysis system (Integrated Semen Analyser System; Proiser, Valencia, Spain). The standard settings of the CASA used correspond to the values of dog spermatozoa defined by the Integrated Semen Analyser System (Proiser). The settings used to define progressive motility are specific to bears and are defined by our previous experience [4]. Samples were diluted (10 to 20 $\times 10^6$ cells per mL) in buffered medium (HEPES 20 mm/L, 197 mmol/L NaCl, 2.5 mmol/L KOH, 10 mmol/L glucose, 1% egg yolk; pH 7; 300 mOsm/kg), and warmed on a 37 °C plate for 5 minutes. Then, a 5 μL sperm sample was placed into a Makler cell counting chamber (10 μm depth; Sefi Medical Instruments, Haifa, Israel). The sample was examined at magnification × 100 (negative phase contrast) in a microscope with a warmed stage (38 °C). The standard parameter settings were set at 25 frames per second, 5 to 50 μm^2 for head area and curvilinear velocity (VCL) >10 μm/s to classify a spermatozoon as motile. At least five sequences or 200 spermatozoa were saved and analyzed afterward. Reported parameters were average velocity (μm/s) and VCL (μm/s). Total motility was defined as the percentage of spermatozoa with VCL >10 μm/s, and progressive motility was defined as the percentage of spermatozoa with VCL >25 μm/s and straightness >80% (also provided by the system).

2.4.2. Flow cytometric evaluation

Viability and acrosomal status were assessed using the double stain PNA-FITC/propidium iodide (PI) and flow cytometry. Stock solutions of the fluorochromes were prepared in PBS at 1 μg/mL PNA-FITC and 1.5 μM PI. Sperm samples were diluted with fluorochrome solution down to 5×10^6 spermatozoa per mL in polypropylene tubes (300 μL per tube). The flow cytometry analysis yielded the percentage of spermatozoa viable and with intact acrosomes (PI−) and the percentage of spermatozoa with damaged acrosomes (PNA-FITC+).

Early changes in plasma membrane permeability was assessed with 0.1 μM YO-PRO-1 and 1.5 μM PI. This probe allows us to evaluate the increment in membrane permeability that usually occurs after refrigeration or cryopreservation processes.

Flow cytometry evaluations were carried out using a FACScalibur flow cytometer (Becton Dickinson BioSciences,

San Jose, CA, USA), equipped with standard optics and an argon-ion laser, tuned at 488 nm and running at 200 mW. Calibration was carried out periodically using standard beads (Calibrites; Becton Dickinson). Data corresponding to the red (FL3 photodetector; PI) and green fluorescence (FL1 photodetector; YO-PRO-1 and FITC) of acquired particles were recorded. In all the cases we assessed 10,000 events per sample, with a flow rate of 200 cells per second.

2.5. Statistical analysis

Data were analyzed using the SAS V.9.1. package (SAS Institute Inc., Cary, NC, USA). Results are shown as mean

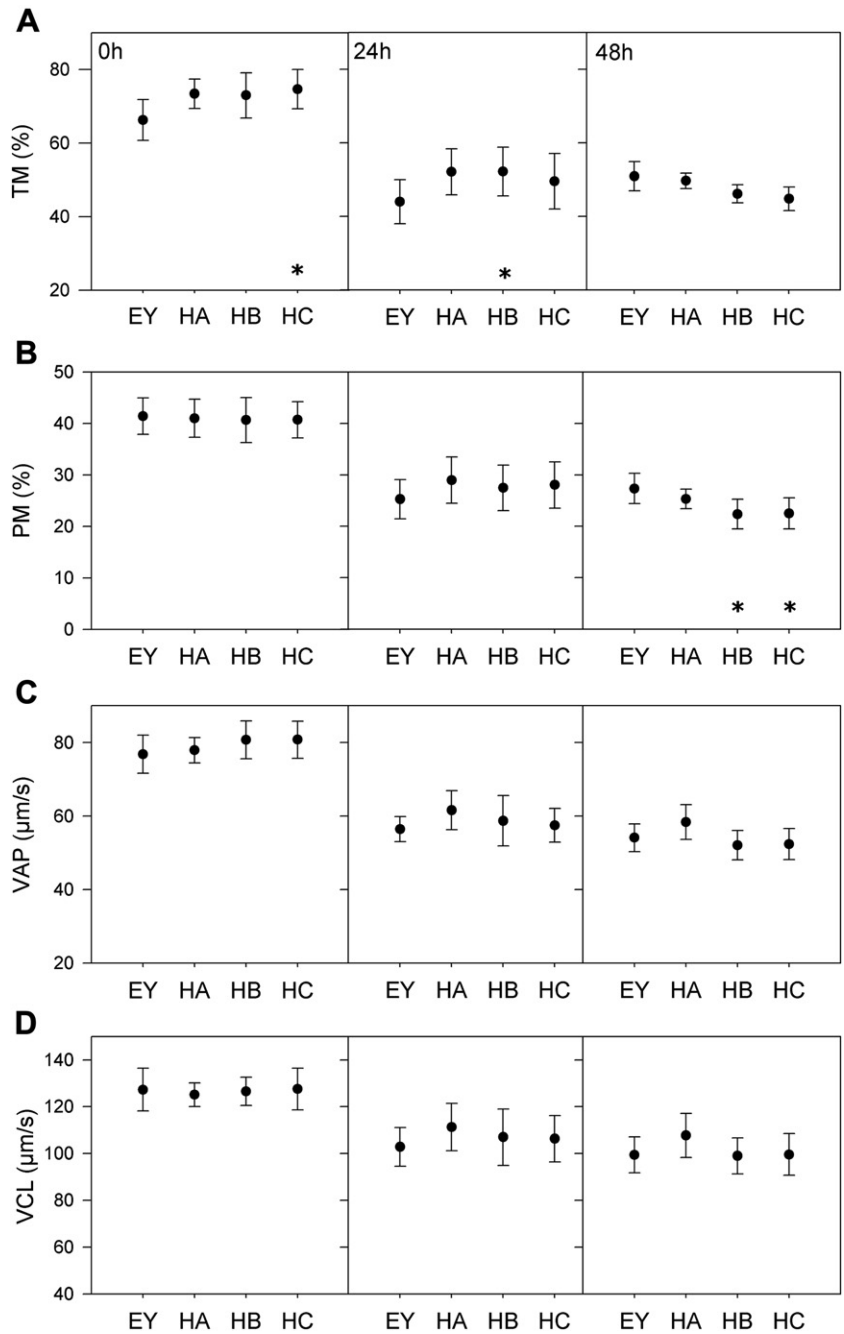

Fig. 1. Motility parameters for the use of heat shock protein A8 (HSPA8) protein in the refrigeration storage (5 °C) of semen samples (mean ± SEM). Extenders: TES-TRIS-fructose-20% egg yolk with 0 μg/mL HSPA8 (EY), EY with 0.5 μg/mL HSPA8 (HA), EY with 1 μg/mL HSPA8 (HB), and EY with 5 μg/mL HSPA8 (HC). Time represents the refrigeration time (hours) of the samples at 5 °C (0, 24, and 48 hours). Motility parameters: total motility (TM; %), progressive motility (PM; %), average velocity (VAP; μm/s), and curvilinear velocity (VCL; μm/s). * Differences ($P < 0.05$) respect to the control (EY).

and standard error of the mean, unless otherwise stated. Residuals were tested for normality (Shapiro–Wilk test). The data of progression tests were normally distributed. Percentage data were arcsin square-root transformed when necessary. Analyses of the prefreezing, postthawing, and migration test data were carried out using linear mixed-effects models (MIXED procedure, ML method). Models were built with (1) refrigeration storage variables (concentrations of HSPA8 and storage time) or (2) freezing-thawing variables (concentrations of HSPA8 and test time) or (3) progression test variables (concentrations of HSPA8) as fixed effect and males and samples in the random part of the model (random effect). Significant fixed effects were further analyzed using multiple comparisons of means with Tukey contrasts. A significance level of $P < 0.05$ was used.

The relationships between the result of the vanguard spermatozoon test and the HSPA8 concentration in extender was analyzed using a univariate test of significance for planned comparison with some specific contrasts within the ANOVA procedure.

3. Results

The fresh Brown bear semen samples showed the following values for volume, pH, osmotic pressure, and cellular concentration (mean ± SEM): 4.5 ± 1.0 mL, 8.00 ± 0.20, 294.3 ± 4.9 mOsm/kg, and 229.3 ± 88.5 × 10^6 spermatozoa per mL.

3.1. Experiment 1. Use of HSPA8 in the refrigeration storage (5 °C) of semen samples

Motility parameters (Fig. 1) showed higher values (HC with respect to the control; $P < 0.05$) at 0 hours for total motility (Fig. 1A) and 24 hours in HB. Progressive

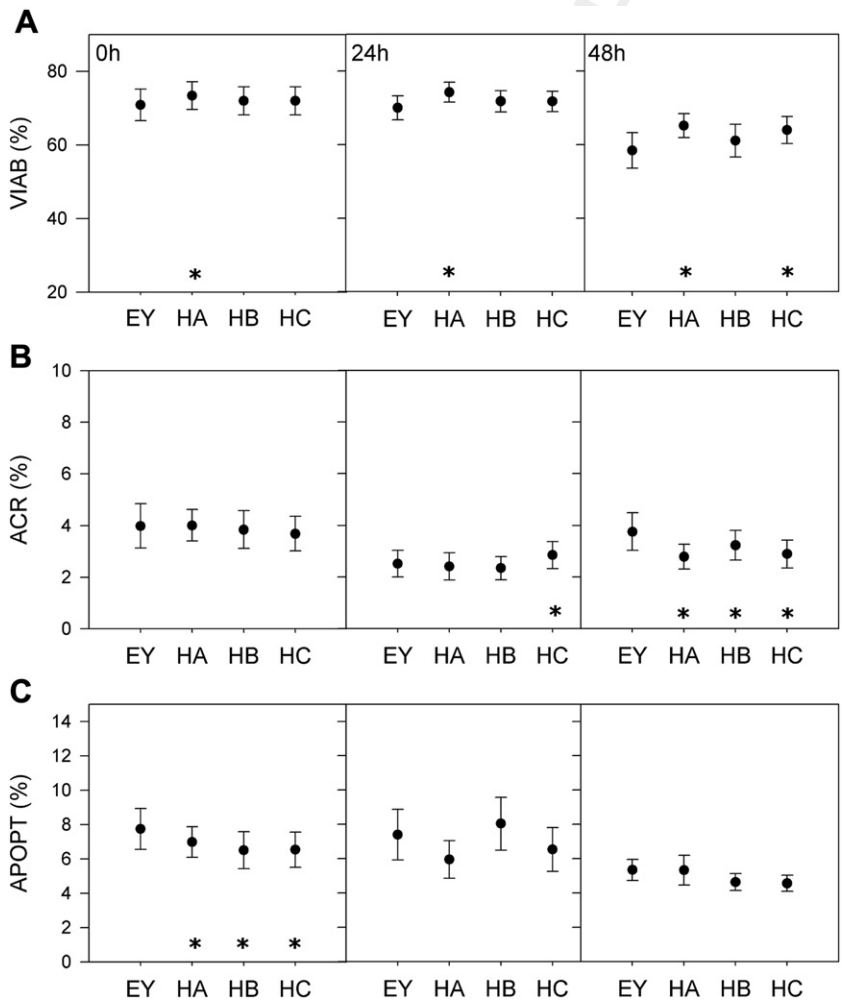

Fig. 2. Flow cytometry parameters for the use of heat shock protein A8 (HSPA8) protein in the refrigeration storage (5 °C) of semen samples (mean ± SEM). Extenders: TES-TRIS-fructose-20% egg yolk with 0 μg/mL HSPA8 (EY), EY with 0.5 μg/mL HSPA8 (HA), EY with 1 μg/mL HSPA8 (HB), and EY with 5 μg/mL HSPA8 (HC). Times represent the refrigeration time (hours) of the samples at 5 °C (0, 24, and 48 hours). Flow cytometry analysis: viable and with intact acrosomes (VIAB) (propidium iodide [PI]−); viable sperm and acrosomes (ACR) (PNA-FITC+); number of damaged acrosomes and changes to membrane permeability (APOPT) (YO-PRO-1+); sperm with early changes in their membrane (apoptotic-like). * Differences ($P < 0.05$) with respect to the control (EY).

motility at 48 hours (Fig. 1B) showed lower values for HB and HC.

In addition, viability (Fig. 2A) showed higher values in HA at 0, 24, and 48 hours ($P < 0.05$), and also HC at 48 hours, with respect to the control. Number of sperm with damaged acrosomes (Fig. 2B) showed lower values ($P < 0.05$) for all concentrations of protein at 48 hours and for HC at 24 hours. Apoptotic index measured with YO-PRO-1 (Fig. 2C) showed lower values at 0 hours for all concentrations of HSPA8 ($P < 0.05$).

3.2. Experiment 2. Use of HSPA8 in the cryopreservation of semen samples

Prefreezing motility parameters (Fig. 3) and membrane status (Fig. 4) showed no differences.

Also, no differences in postthaw motility parameters were found (Fig. 3), but early changes to membrane permeability showed lower values for HB after a 37 °C incubation test over 2 hours (Fig. 4C) In contrast, immediately after thawing HC showed higher value of changes to membrane permeability. Moreover, HB and HC showed lower values after the incubation test.

3.3. Experiment 3. Assessment of frozen-thawed spermatozoa using a hyaluronic acid migration test

Results for the maximum distance that one single spermatozoon was able to travel showed that the lower HSPA8 concentration significantly reduced the distance traveled by the vanguard sperm with respect to control (Fig. 5A) but that the higher HSPA8 concentrations restored the progressive ability of this sperm with respect to the control. In contrast, univariate tests of the vanguard spermatozoon results (Fig. 6) showed a significant linear relationship (dose-dependent) between HA, HB, and HC ($P < 0.05$).

The total number of sperm measured in a specific distance (from 0.5 cm to 2 cm) (Fig. 5B) showed a significant decrease of the number of spermatozoa for all the concentrations of HSPA8, with respect to the control.

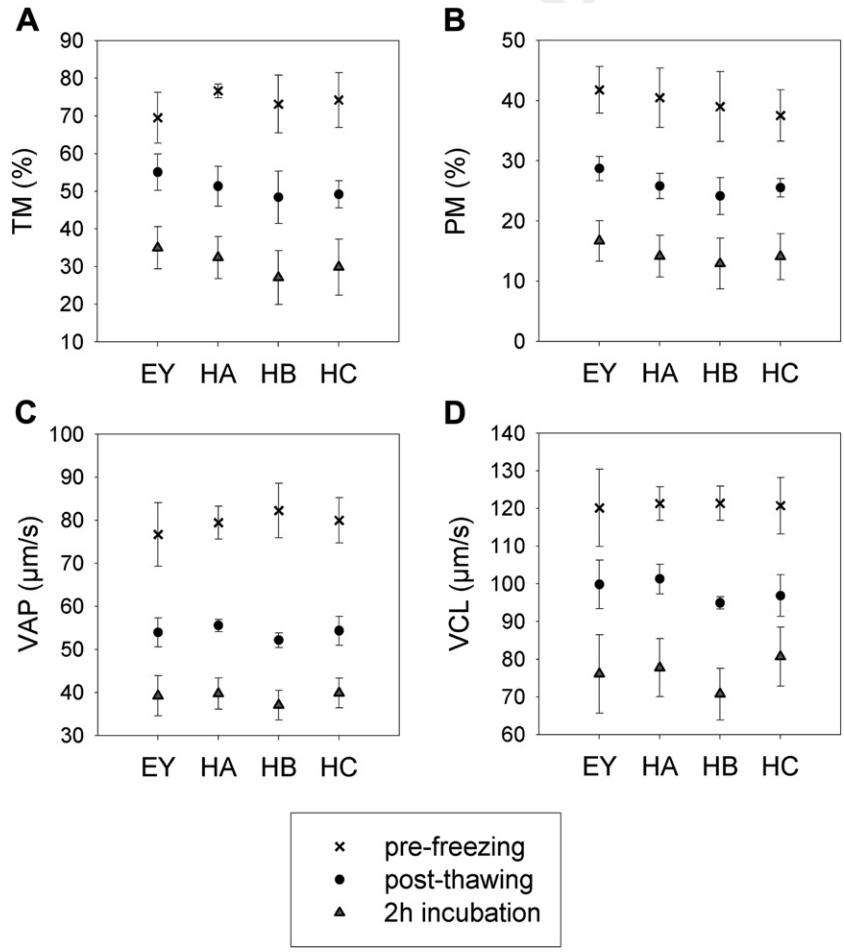

Fig. 3. Motility prefreezing, postthawing, and 2-hour incubation (37 °C) parameters for the use of heat shock protein A8 (HSPA8) in the cryopreservation of semen samples (mean ± SEM). Extenders: TES-TRIS-fructose-20% egg yolk with 0 µg/mL HSPA8 (EY), EY with 0.5 µg/mL HSPA8 (HA), EY with 1 µg/mL HSPA8 (HB), and EY with 5 µg/mL HSPA8 (HC). Motility parameters: total motility (TM; %), progressive motility (PM; %), average velocity (VAP; µm/s), and curvilinear velocity (VCL; µm/s). * Differences ($P < 0.05$) with respect to the control (EY).

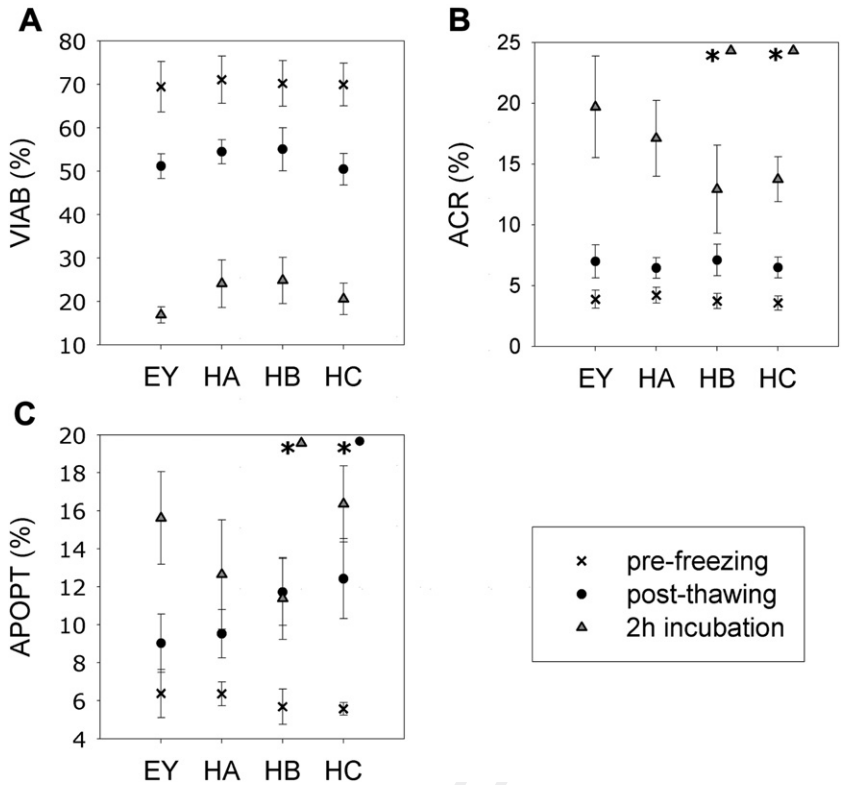

Fig. 4. Flow cytometry prefreezing, postthawing, and 2-hour incubation (37 °C) parameters for the use of heat shock protein A8 (HSPA8) in the cryopreservation of semen samples (mean ± SEM). Extenders: TES-TRIS-fructose-20% egg yolk with 0 μg/mL HSPA8 (EY), EY with 0.5 μg/mL HSPA8 (HA), EY with 1 μg/mL HSPA8 (HB), and EY with 5 μg/mL HSPA8 (HC). Flow cytometry analysis: viable and with intact acrosomes (VIAB) (propidium iodide [PI]−) viable sperm, acrosomes (ACR) (PNA-FITC+); number of damaged acrosomes and changes to membrane permeability (APOPT) (YO-PRO-1+); sperm with early changes in their membrane (apoptotic-like). * Differences ($P < 0.05$) respect to the control (EY).

4. Discussion

This study evaluated the semen quality after of the use of HSPA8 protein (0.5, 1, and 5 μg/mL) as an additive in the refrigeration and cryopreservation of Brown bear sperm samples in order to improve the sperm preservation in this species. Moreover, we carried out a sperm progression test to evaluate the effect of protein addition on sperm motility.

In the first experiment, progressive motility showed a significant decrease at 48 hours of refrigeration after the use of HSPA8 protein, but HSPA8 showed also a significant enhancement of viability at the lower concentration 0.5 μg/mL for 0, 24, and 48 hours of refrigeration storage, and by 48 hours the number of acrosomes damaged was significantly reduced for all concentrations. The effect on Brown bear sperm refrigeration was consistent with the results obtained for other species: boar and bull (studies carried out at 38 °C) [16] and ram (studies carried out at 5 °C and 38 °C) [17]. We interpret this result as being a consequence of the conserved nature of this protein across species [18]. Heat shock protein A8 is a member of the HSP70 family of proteins (70 kDa), and is regarded as among the most conserved protein families in evolution. It is found in all organisms from archabacteria and plants to humans, and the prokaryotes.

One of the objectives of the present study was to assay the protective activities of the protein by exogenous addition and to optimize the amount of protein needed for sperm quality preservation. Results from the refrigeration study were encouraging and showed a beneficial effect on viability that confirmed the potential role of HSPA8 on maintaining the quality of Brown bear samples. There were few improvements of the flow cytometry parameters when these were assessed immediately after thawing, but the 2-hour incubation stress test revealed that the HSPA8 significantly protected the sperm plasma membrane against permeabilization (HB extender) and reduced the proportion of sperm with damaged acrosomes (HB and HC extender). These results suggest improved survival of frozen-thawed spermatozoa in the female reproductive tract, like the study on cryopreserved ram sperm which concluded that the addition of seminal plasma proteins increased the sperm resistance to damage from cold shock [21]. In this sense, the severely shortened lifespan of cryopreserved spermatozoa within the female tract is a major cause of poor fertility and the ability of HSPA8 to protect spermatozoa against cold injury could help improve this result.

Spermatozoa are not capable of synthesizing heat shock proteins (HSPs) in response to stress, unlike somatic cells, because ejaculated spermatozoa are transcriptionally inactive. Therefore, they depend on preformed HSPs for

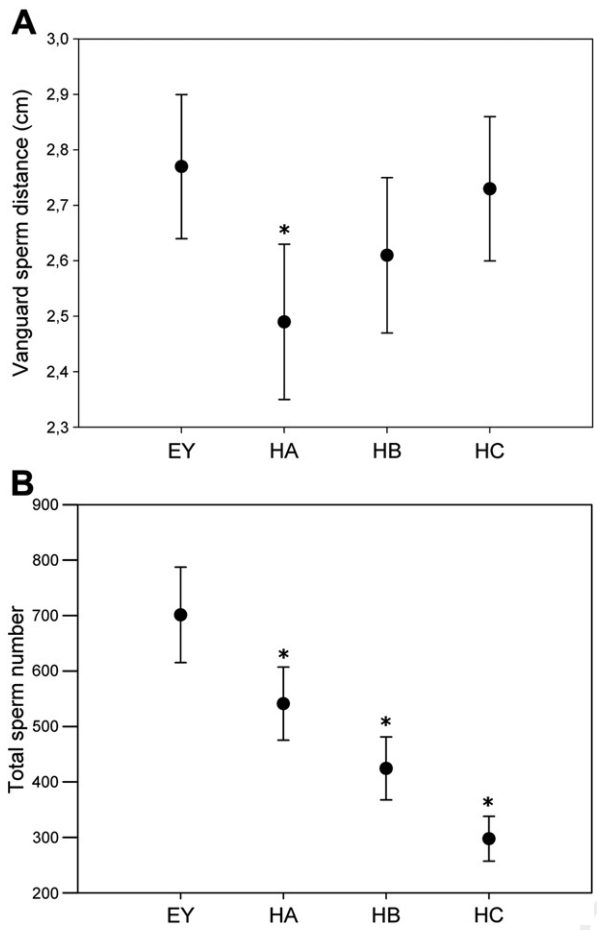

Fig. 5. Data (mean ± SEM) of migration test of frozen-thawed sperm through artificial mucus (hyaluronic acid). Extenders: TES-TRIS-fructose-20% egg yolk with 0 μg/mL HSPA8 (EY), EY with 0.5 μg/mL HSPA8 (HA), EY with 1 μg/mL HSPA8 (HB), and EY with 5 μg/mL HSPA8 (HC). (A) Vanguard spermatozoon (the linear distance covered by the foremost sperm cell) and (B) total number of sperm counted between 0.5 cm and 2 cm of capillary tube. * Differences ($P < 0.05$) with respect to the control (EY).

protection against various stresses [22]. But the HSP might be outside the sperm, being produced by the oviduct. Thus, it has been reported that when sperm reach the oviduct they stimulate the upregulation of HSPA8 and that it is translocated into the extracellular environment (the lumen of the oviduct) [23]. This extracellular HSP activity has been increasingly recognized and studied over the past few years [15,24]. In addition, it seems that HSPA8 interacts with lipid rafts and cholesterol, and this might be important in terms of protecting the sperm [25].

On the other hand, another possible role of the HSP70 family is inhibition of the accumulation of protein aggregates, thus removing the stimulus that might constitute an apoptosis signaling pathway for the cells [26]. The early changes in plasma membrane permeability (apoptotic-like changes) obtained in refrigeration storage (experiment 1), showed lower value for all concentrations at 0 hours and a slight tendency to decrease the apoptotic index at the other refrigeration times (24 and 48 hours). In contrast, the

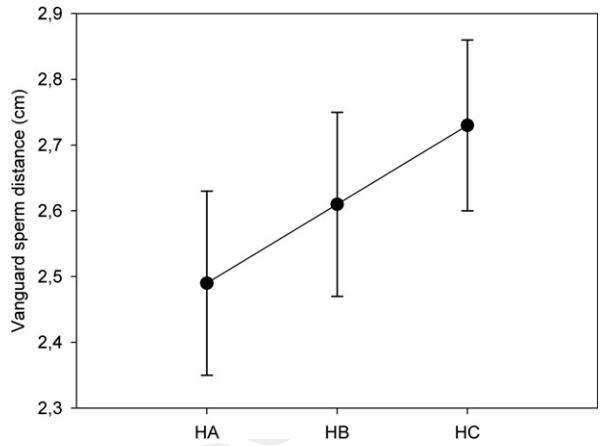

Fig. 6. Linear distance covered by the vanguard spermatozoon (foremost sperm cell) (mean ± SEM) obtained in the migration test of frozen-thawed sperm through artificial mucus (hyaluronic acid) analyzed with the extenders, TES-TRIS-fructose-20% egg yolk with 0.5 μg/mL heat shock protein A8 [HSPA8] (HA), HA with 1 μg/mL HSPA8 (HB), and HA with 5 μg/mL HSPA8 (HC). Line represents a significant linear relationship between HA, HB, and HC (univariate test with specific contrast, $P = 0.0077$).

cryopreservation process showed no clear tendency to decrease the apoptotic index, except after the thermal stress test in which we obtained a protective effect of 1 μg/mL, whereas the higher concentration produced a detrimental effect on sperm immediately after thawing. In terms of number of acrosomes damaged, results showed a significant decrease in values for both HB and HC after the incubation test. One explanation of these differences could be that the constant changes in sperm membrane physiology during this process, together with the egg yolk and glycerol present in the extender could interfere in the protein activity. In this regard, a freezing experiment without egg yolk and glycerol might be a good assay to test the isolated effect that the protein could have on sperm protection during cryopreservation.

We carried out a simple test using hyaluronic acid in order to see whether the presence of HSPA8 might improve the ability of spermatozoa to migrate through a viscous medium. Hyaluronic acid was chosen for this experiment because some authors have suggested that normal spermatozoa in other species have hyaluronic acid-binding receptors (CD44) [27]. Moreover, hyaluronic acid has been shown to stimulate human sperm motility by increasing intracellular Ca^{2+} concentration [28]. In practical applications, studies carried out with human spermatozoa showed that effective selection of spermatozoa without DNA fragmentation could be achieved using hyaluronic acid binding [29] and that there are some beneficial effects on ICSI outcomes if hyaluronic acid was used in a sperm selection step [30]. Q6

The effects of hyaluronic acid sperm selection in this experiment were especially interesting because there were two different and distinct outcomes. As a whole sperm population the functional motility was systematically suppressed by the presence of HSPA8. This effect was significantly dose-dependent and was detectable at the lowest dose tested. Paradoxically, however, the maximum distances traveled within the capillary tubes by the

vanguard sperm revealed the opposite tendency. A very clear positive relationship between maximum distance and the HSPA8 concentration was observed; this implies that a distinctive but small sperm subpopulation present within the bulk samples could retain its functional swimming ability when cryopreserved in the presence of 1 and 5 μg/mL of HSPA8. This is not unlike the situation inside the oviduct, where most sperm are not highly active, and a small number are sufficiently active to progress toward the oocyte for fertilization [11]. These effects could also be because of selective blocking by HSPA8 of the passage of the poorer motility sperm, and allowing some high motility sperm to enhance their motility. This interpretation is consistent with the reduced polyspermy rate that is found in porcine IVF when spermatozoa are exposed to HSPA8 before, or during, IVF [16].

The effect of the HSPA8 protein might therefore be related to a poorly defined mechanism that allows spermatozoa to remain within the sperm reservoir in the female tract. The protein reduces the motility of the overall sperm population, but the vanguard sperm must represent a very special subpopulation, which can be "rescued" by the protein. In this sense, the nonmotile sperm could be differentially sensitive to inhibitory signaling activity from the HSPA8 [31]. But further studies should be carried out to determine whether the effect of HSPA8 might be linked to the establishment and maintenance of the oviductal sperm reservoir.

In vitro tests involving sperm migration through capillary tubes have been used previously to examine sperm quality in several species: in goats, Cox et al. [19] observed a correlation between migration distance and the ability to colonize the oviduct and penetrate eggs during *in vitro* fertilization. These authors also found that sperm migration efficiency in homologous mucus could be related to velocity parameters, linearity and lateral head displacement of the vanguard sperm population. Other authors (bull [32]; bovine [33]) have suggested that there is no relationship between mucus penetration and field fertility, but these results could be influenced by the use of methyl cellulose instead of hyaluronic acid [32] and the elaboration of pools of samples that varied between 10 and 210 straws per sample for each insemination [33].

4.1. Conclusions

We have presented some preliminary evidence to show that low concentrations of HSPA8 (0.5 μg/mL) could be useful for improving existing diluents for Brown bear semen refrigeration storage. Postthawing results showed a beneficial effect of the protein in improving the acrosomal status, and also a protective effect of HSPA8 (1 μg/mL) on sperm submitted to a thermal stress test (37 °C during 2 hours). The thermal stress response was improved and the migration efficiency of a small population was also improved by the addition of HSPA8 to Brown bear sperm extender.

Acknowledgments

This work was supported in part by MICINN (CGL 2010-19213/BOS) and CANTUR S.A. Felipe Martinez-Pastor was supported by the Ramón y Cajal program (RYC-2008-02560, MICINN, Spain). The authors thank Miguel Ángel Marañón and the game keepers of the Cabárceno Nature Park, María Mata Campuzano, Julio Tamayo Canul, Leticia Ordás Bandera, Carmen Martinez Rodríguez, Susana Gomes Alves, María Nicolás, Elena López Urueña, and Patricia Manrique Revuelta and Alireza Fazelis's lab members of the Department of Human Metabolism in Sheffield, for their help in the artificial mucus test.

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