Heat-shock protein A8 restores sperm membrane integrity by increasing plasma membrane fluidity

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Abstract

The constitutive 70 kDa heat-shock protein, HSPA8, has previously been shown to contribute to the long-term survival of spermatozoa inside the mammalian female reproductive tract. Here, we show that a recombinant form of HSPA8 rapidly promotes the viability of uncapacitated spermatozoa, the ability of spermatozoa to bind to oviductal epithelial cells, enhances IVF performance, and decreases sperm mitochondrial activity. Fluorescence recovery after photobleaching revealed that the repair of membrane damage is achieved by an almost instantaneous increase in sperm membrane fluidity. The ability of HSPA8 to influence membrane stability and fluidity, as well as its conserved nature among mammalian species, supports the idea that this protein protects sperm survival through membrane repair mechanisms.

Free Persian abstract

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Introduction

Oviductal sperm storage plays a key role in reproduction by providing a secure reservoir in which spermatozoa can attain full fertilizing properties. The oviductal epithelium and its secretions influence a number of sperm characteristics, the most notable of which are viability, motility, and capacitation (Grippio *et al.* 1995, Fazeli *et al.* 2003). These characteristics combine to enable spermatozoa to fertilize the oocyte under optimal conditions. A number of protein factors in oviductal fluid and the oviductal apical epithelial membrane have now been shown to affect sperm function. For example, a bovine oviduct-specific glycoprotein, which is secreted by oviductal epithelial cells, effectively maintains the viability and motility of spermatozoa (Abe *et al.* 1995). Proteomic analysis of porcine oviductal fluid has revealed that the oviductal epithelial cells secrete a number of molecules in response to the presence of spermatozoa, most notable of which are heat-shock (stress) proteins (HSPs; Georgiou *et al.* 2005).

HSPs are families of functionally related proteins that are highly conserved in divergent organisms (Burdon 1986, Schlesinger 1986, 1990). HSPs are commonly considered as being molecular chaperones that perform a suite of

intracellular homeostatic housekeeping functions that are focused on the normal functioning of cells and their protection against potentially damaging challenges. These proteins also play central roles in cellular growth and development, as well as in cell metabolism and signal transduction pathways (Picard *et al.* 1990, Schlesinger 1990, Young *et al.* 2001). For many years, the established dogma was that mammalian HSPs are exclusively intracellular molecules and that they are only present in extracellular compartments in pathological conditions such as necrotic cell death. However, overwhelming evidence now points to the concept that stress proteins can be released under non-pathological conditions and have a protective role (Tytell *et al.* 1986, Hightower & Guidon 1989). For example, early research demonstrated the transfer of HSPA1A (HSP70) and HSPA8 (HSC70) from adjacent glial cells to the squid giant axon (Tytell *et al.* 1986) and that the exogenous HSPA1A enhanced the stress tolerance of neuronal cells. It has been suggested that this transfer of HSPs might be part of an altruistic response on the part of one cell for the protection of adjacent cells (Guzhova *et al.* 2001).

Consistent with other reports on protective effects of exogenous HSPs in different cell systems, previous

reports from our laboratory and others have indicated that HSPA8 significantly enhances the *in vitro* survival of boar, bull (Elliott *et al.* 2009), and ram (Lloyd *et al.* 2009) spermatozoa after long-term (24 and 48 h) exposure to HSPA8. However, the mechanism underlying this protective action of HSPA8 on spermatozoa and potential cofactors needed for HSPA8 to convey this effect are not yet known. Furthermore, in all the studies performed to date, the protective effect of exogenous HSPA8 on spermatozoa has been studied following at least 24 h of exposure to HSPA8. It is therefore not clear if the ability of HSPA8 to maintain sperm viability results from protection against damage during *in vitro* culture or the immediate repair of damaged cell membranes and the restoration of sperm viability and membrane integrity via an unknown mechanism.

In the current investigation, we characterized the immediate impact of exogenous HSPA8 on spermatozoa. Our experiments suggest that exogenous HSPA8 is a 'rapid response' extracellular cytoprotector and modifier of cell function, which rapidly restores cell membrane integrity by influencing membrane microviscosity via a mechanism that is dependent on membrane cholesterol.

Materials and methods

Production, overexpression, and purification of HSPA8

PCR amplification of full-length bovine HSPA8 (GenBank accession number NM_174345) from bovine uterine cDNA was performed using the following oligonucleotides: forward, 5'-C ACC ATG TCT AAA GGA CCT GCA GTT-3'; reverse, 5'-ATC AAC CTC TTC AAT GGT GGG-3'. Pfx DNA polymerase was employed to create a blunt-ended PCR product and allowed directional cloning into pET101/DTOP0 (Invitrogen, K101-01) with a C-terminal polyhistidine tag. The presence and correct orientation of the insert was confirmed by double-restriction digest with *Ap*I and *Eco*NI. Inducible expression under the control of the T7lac promoter was performed in BL21 Star (DE3) cells. Bacterial cell lysates containing His-tagged recombinant HSPA8 were applied to Probond nickel chelate resin (Invitrogen) under native conditions. Protein was eluted using 500 mM imidazole and dialyzed into PBS using 10 kDa molecular weight cut-off dialysis membrane. SDS-PAGE and western blot analysis was performed to verify the nature and purity of the recombinant HSPA8.

Immobilized metal ion affinity chromatography-purified HSPA8 (80–2000 ng) was run out under denaturing/reducing conditions on 4–12% w/v Bis-Tris NuPAGE gel in 1× MOPS running buffer at 200 V for ~50 min. Proteins were electrotransferred to 0.2 µm nitrocellulose using 1× transfer buffer at 30 V for 1 h. Nitrocellulose membranes were then blocked in 5% w/v dried skim milk for 2 h and probed with anti-HSPA1A (SPA-810; 1/1000 in 1% w/v skimmed milk powder) or anti-HSPA8 (SPA-815; 1/1000 in 1% w/v skimmed milk powder) for 90 min. After washing for 15 min in 0.05% v/v PBS-Tween, the membranes were probed for 90 min with HRP-conjugated goat anti-mouse (Bio-Rad 172-1011; 1/2000) and rabbit anti-rat (Sigma; A5795; 1/2000) antibodies for HSPA1A and HSPA8

respectively. After washing membranes for 30 min in PBS-Tween with four changes, the signal was visualized using DAB/Urea peroxidase substrate (Sigma D4418) and captured using a digital camera. For the anti-His western blots, membranes were probed with HRP-conjugated anti-His (C-terminus) reagent (Invitrogen; 46-0284) before being developed with DAB/urea peroxidase substrate.

Semen preparation

Boar semen that had been diluted and stored for 24 h in Beltsville Thawing Solution (Pursel & Johnson 1975) was obtained from a commercial artificial insemination station (JSR Healthbred Limited, Thorpe Willoughby, Yorkshire, UK). On the day of each experiment, diluted boar semen was washed using a Percoll gradient (GE Healthcare Bio-Sciences AB, Uppsala, Sweden; Holt & Harrison 2002). For this, 2–4 ml aliquots of diluted semen were layered over an iso-metric Percoll density gradient consisting of 2 ml 70% v/v Percoll overlaid with 2 ml 35% v/v Percoll. The gradient was centrifuged at 200 *g* for 15 min followed by 15-min centrifugation at 1000 *g*. The supernatant was removed and the pellet was resuspended in modified Tyrodes medium containing, 3.1 mM KCl, 0.4 mM MgCl₂·6H₂O, 2 mM CaCl₂, 25 mM NaHCO₃, 10 mM HEPES, and 0.3 mM NaH₂PO₄·2H₂O. The sperm concentration was determined using a hemocytometer and adjusted to 5×10⁶ spermatozoa/ml in TALP medium that consisted of Tyrodes medium supplemented with 12 mg/ml BSA, 21.6 mM sodium lactate, and 1 mM sodium pyruvate. All chemicals used for preparation of TALP medium were purchased from BDH Laboratory Supplies, Poole, UK.

Assessment of sperm viability

Sperm viability was evaluated using a combination of ethidium homodimer/calcein-AM (Viability/Cytotoxicity kit: Molecular Probes, Eugene, OR, USA) and Propidium iodide/SYBR-14 (Live/Dead Sperm Viability Kit, Molecular Probes) viability staining assays. For this, ethidium homodimer and calcein-AM (0.4 and 0.08 µM) and propidium iodide and SYBR-14 (12 µM, 4 nM) were added to 100 µl semen aliquots (5×10⁶ spermatozoa/ml). Samples were mixed and incubated for 30 min at 39 °C in 5% v/v CO₂. An aliquot (10 µl) of each semen sample was placed on a slide and evaluated using a fluorescence microscope (Olympus BH2, Olympus) at 400× magnification using a dual rhodamine-FITC filter. Three slides were prepared for each sample and a minimum of 200 spermatozoa were evaluated per slide. Green fluorescent spermatozoa were classified as live and red fluorescent spermatozoa were classified as dead.

Chlortetracycline hydrochloride capacitation assay

The capacitation status of boar sperm was assessed using the chlortetracycline hydrochloride (CTC) staining method, as described by Fazeli *et al.* (1999). Following CTC staining, 200 spermatozoa per slide (three slides for each sample) were immediately evaluated using an epifluorescence microscope (Olympus BH2, Olympus) at 400× magnification. The CTC assay revealed three patterns of uniform fluorescence over the

whole head (uncapacitated), a fluorescence-free band in the postacrosomal region (capacitated), and no fluorescent over the whole head (acrosome reacted).

Assessment of mitochondrial activity

Spermatozoa were stained with JC-1 mitochondrial probe (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbo-cyanine iodide; Sigma–Aldrich) according to the manufacturer's instructions and its proprietary staining buffer. Aliquots of Percoll-washed sperm (200 μ l, 5×10^6 /ml) were incubated for 20 min with 200 μ l staining buffer containing 7.6 nM JC-1 and 12 μ M propidium iodide at 37 °C in a humidified atmosphere containing 5% v/v CO₂. The suspension was centrifuged at 600 *g* for 3–4 min at 2–8 °C. The pellet was washed with staining buffer and resuspended in the same solution prior to microscopic evaluation. Evaluation was performed by epifluorescence microscope (Olympus BH2, Olympus) at 400 $\times$ magnification.

Sperm-OEC binding assay

Equal volumes of Percoll-washed boar semen samples and pig primary oviduct epithelial cells were incubated at 39 °C, 5% v/v CO₂ for 30 min on a rotator. Unbound or loosely attached spermatozoa were removed by layering 400 μ l of the sperm-oviductal epithelial cell suspension over a two-step Percoll gradient containing 1 ml 35% w/v Percoll overlaid on the top of 1 ml 70% w/v Percoll in a 15 ml polypropylene tube. After centrifugation at 200 *g* for 3 min, three layers of cells appeared. Non-motile, unbound spermatozoa and unattached oviductal epithelial cells located at the interface between the media and the 35% w/v Percoll layer (layer 1). Sperm-oviductal epithelial cell complexes located at the interface of the two Percoll layers (layer 2) and unattached highly motile spermatozoa that had sedimented to the bottom of the tube (layer 3). Cells from layers 1 and 2 were carefully removed using a pipette, combined, and diluted in 15 ml PBS. Unattached spermatozoa were removed by centrifugation at 200 *g* for 5 min. The supernatant was discarded and sperm-oviductal epithelial cells were resuspended in 500 μ l PBS and fixed with 1% w/v formaldehyde in PBS prior to microscopic evaluation (Green *et al.* 2001).

Effect of HSPA8 on IVF capacity

Ovaries from prepubertal gilts were transferred from the local slaughterhouse to the laboratory in 0.9% v/v NaCl containing 70 μ g/ml kanamycin, at 34–37 °C within 1 h of collection. Ovaries were gently washed three times in prewarmed (37 °C) NaCl solution and follicles of 3–6 mm in diameter were collected by making gentle cuts on the ovarian wall. Released oocytes with compact cumulus mass and dark evenly granulated cytoplasm were washed three times in maturation medium and 70–80 oocytes in 500 μ l maturation medium supplemented with 10 IU/ml equine chorionic hormone (eCG, Folligon, Intervet International B.V., Boxxmeer, The Netherlands) and 10 IU/ml human chorionic hormone (hCG, Veterin Corion, Divasa Farmavic, S.A., Barcelona, Spain) were transferred into each well of a four-well plate. These were

incubated at 39 °C, 5% v/v O₂ under mineral oil for 22 h and then for another 22 h in maturation medium without hormones for *in vitro* maturation (IVM).

After IVM, cumulus–oocyte complexes were stripped of cumulus cells by briefly vortexing with 0.1% w/v hyaluronidase in maturation medium. Denuded oocytes were then washed twice in maturation medium and three times in pre-equilibrated fertilization medium (modified Tris-buffered medium, mTBM) consisting of 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl₂·2H₂O, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, 0.2% w/v BSA (fraction V; Sigma 7888), and 0.5 mM caffeine (Abeydeera & Day 1997). Groups of 50 denuded oocytes were then placed in 50 μ l droplets of fertilization medium in four-well plate dishes under mineral oil at 39 °C in 5% v/v CO₂ in air. After 30 min, fresh boar spermatozoa were diluted to the appropriate concentration for insemination and 50 μ l diluted spermatozoa were added to oocytes in IVF droplets to give a final concentration of 25 000 spermatozoa (sperm:oocyte ratio, 500:1).

Five hours post-insemination, presumptive zygotes were washed three times in pre-equilibrated embryo culture medium (North Carolina State University (NCSU-23): 108.73 mM NaCl, 4.78 mM KCl, 1.19 mM K₂PO₄, 1.19 mM MgSO₄·7H₂O, 25.07 mM NaHCO₃, 1 mM L-glutamine, 7 mM Taurine, 5 mM hypotaurine, penicillin, and streptomycin (Petters & Wells 1993) supplemented with 0.4% w/v BSA (fraction V) for the first 2 days of embryo culture and with NCSU-23 containing 5.55 mM D-glucose thereafter. At this stage, vigorous washing removed attached spermatozoa from oocytes. Groups of 50 washed zygotes were transferred to four-well dish containing 500 μ l culture medium under mineral oil. Plates were cultured for 16 h at 39 °C in 5% v/v CO₂.

At the end of the culture period, 50% of the zygotes were fixed on slides in acetic acid:ethanol solution at 1:3 (v/v) ratio for 48–72 h at room temperature for the assessment of fertilization parameters. Fixed oocytes were then stained with 1% v/v lacmoid in 45% v/v acetic acid and examined for fertilization parameters under a phase-contrast microscope (Eclipse E400, Nikon, Japan) at 200 $\times$ and 400 $\times$ magnification. The rest of the oocytes were continued in culture until 48 h, at which time they were transferred to fresh culture medium to be studied for early embryonic development parameters on the 2nd and 7th days after IVF.

Fluorescence recovery after photobleaching

Fluorescence recovery after photobleaching (FRAP) was used to assess sperm membrane fluidity. FRAP is an optical technique that measures the diffusion and recovery of fluorescent lipid probe, which is inserted into the membrane bilayer after laser-induced photobleaching of membrane. The lipid probe 5-(N-octa-decanoyl) amino fluorescein (ODAF, excited at 488 nm) was purchased from Molecular Probes.

For the labeling, equal volumes of Percoll-washed boar semen in Tyrodes (5×10^6 /ml) and 12.5 μ M ODAF in 2% v/v ethanol were incubated for 15 min at room temperature, after which they were washed twice in Tyrodes by centrifugation at 400 *g* for 10 min (Wolfe *et al.* 1998).

The appearance of live and dead cells is markedly different in samples stained with ODAF; live spermatozoa are always

stained weakly, whereas the dead and membrane-disrupted ones absorb more ODAF and exhibit a stronger fluorescence, particularly over the acrosome and mid piece domains (Wolfe *et al.* 1998). The distinction between live and dead cells enabled the examination of the effect of HSPA8 on the membrane fluidity of live spermatozoa only. ODAF uptake was monitored by a Zeiss epifluorescence microscope (Carl Zeiss Ltd, London, UK) fitted with a 100 $\times$ objective lens. The incident ~ 2.5 μ m laser beam was generated by a water-cooled argon ion laser and bleaching time was 5 ms at 0.2 mW. FRAP analysis was performed using a COOLSNAPHQ/ICX285 camera (Photometrics, Maidenhead, UK) using 406 nm excitation and 530 nm emission filters. The kinetics of fluorescence recovery included the rate of diffusion (D -values 10^{-9} cm²/s) of the reporter probe within the plane of membrane and level of recovery (R%), which indicated the proportion of freely diffusing reporter molecules (Wolfe *et al.* 1998).

Membrane cholesterol quantification

Sperm membrane cholesterol content was determined using the Cholesterol Liquicolor Enzymatic assay (Stanbio, Boerne, TX, USA). For this, pellets of Percoll-washed or membrane cholesterol-modified spermatozoa were resuspended and washed (600 g, 10 min) in PBS (171 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH 7.4). After a second wash, the sperm pellet was resuspended in 1 ml PBS and the sperm concentration was determined. Samples were stored at -20 °C until the analysis of cholesterol.

After thawing, plasma membranes were solubilized by incubating samples 1:1 (v:v) with lysate buffer (0.4% v/v Triton X-100 in PBS) for 1 h. The same samples were further diluted 1:5 (v:v) with the reagent provided in the kit and incubated for 25 min at 37 °C. Samples were then washed and the supernatant analyzed for cholesterol using spectrophotometry (Cecil Instruments, Cambridge, UK) at 500 nm. Sample cholesterol concentrations were determined using a standard dose–response curve that was generated in parallel.

Depletion of membrane cholesterol

Methyl- β -cyclodextrin (M β CD; Sigma–Aldrich) was used as a facilitating agent for cholesterol efflux. As M β CD as a cholesterol-accepting agent is believed to have toxic effects on cell viability, preliminary experiments were carried out to determine the optimal incubation medium at which the desirable amount of cholesterol was reduced without compromising sperm viability or membrane integrity. As it was observed that cholesterol modification of sperm was largely dependent on the sperm:M β CD ratio and their co-incubation period, the incubation medium and time for reduction of sperm cholesterol was always standardized according to the obtained data, as explained below.

A working solution of M β CD (16 mM) was prepared by dissolving 2 mg M β CD in 1 ml Tyrodes solution in a glass tube. Fresh Percoll-washed spermatozoa were diluted in TLP and treated with 0, 1, 2, 4, and 8 mM M β CD/100 $\times 10^8$ spermatozoa for 30 min at 20–22 °C on a gentle shaker.

The mixture was washed through 45% Percoll gradient at 600 g for 20 min, and the pellet was washed once more in PBS (600 g, 10 min) before being resuspended in TLP. The remaining amount of cholesterol in the spermatozoa was measured as described above.

Repletion of membrane cholesterol

M β CD-loaded cholesterol (CLC) was prepared according to the method described previously (Purdy & Graham 2004, Moore *et al.* 2005). On the day of the experiment, a working solution of 16 mg/ml CLC was prepared in Tyrodes by vigorous vortexing at 37 °C. Spermatozoa were treated with 0, 1, 2, 4, and 8 mg CLC/10⁸ spermatozoa for 30 min at 20–22 °C. The incubation was performed using a gentle shaker in order to allow CLC to distribute and have homogeneous effects on the treated cells. After removing CLC using a 45% Percoll gradient (600 g for 20 min), the amount of cholesterol in the spermatozoa was measured as described above.

Experimental design

Production of recombinant HSPA8 and its effect on boar sperm viability

Recombinant HSPA8 was produced using an *in vitro* *Escherichia coli* recombinant protein production system. Percoll-washed spermatozoa from ten different boars were diluted in TALP and incubated with HSPA8 (0, 0.1, 0.5, and 1 μ g/ml) for 15 min at room temperature, 24 and 48 h at 39 °C, and 5% v/v CO₂. Sperm viability was evaluated microscopically using calcein-AM/Ethidium homodimer (Viability/Cytotoxicity kit: Molecular Probes) and SYBR-14/ propidium iodide (Live/Dead Sperm Viability Kit, Molecular Probes).

Specificity of HSPA8-mediated effects on boar sperm viability

The specificity of the HSPA8-mediated prolongation of sperm survival was assessed by comparing the effects of HSPA8, HSPA1A (human recombinant stress-induced HSPA1A, StressMarq, Victoria, BC, Canada), and α -tubulin (Abcam, Cambridge, UK) on sperm viability. A combination of HSPA1A/HSPA8 was used to determine the potential presence of any synergistic influence on prolonging sperm viability. For these experiments, washed spermatozoa from nine boars were incubated for 15 min in TALP at room temperature containing different proteins: HSPA8 (0, 0.5 μ g/ml), HSPA1A (0.1, 0.5, 1 μ g/ml), and HSPA1A/HSPA8 mixtures (0.1/0.1, 0.5/0.1, 0.5/0.5, and 1/0.5 μ g/ml).

Effect of background-level endotoxin present in recombinant HSPA8 on boar sperm viability

Endotoxin background level present in our in-house-produced recombinant HSPA8 was 300 IU/ml as determined by a semi-quantitative Limulus amoebocyte lysate assay (LAL assay; Sigma–Aldrich). The presence of background endotoxin was expected in recombinant HSPA8 used in our investigation as the recombinant protein was produced in an *E. coli* over-expression system. To rule out the potential detrimental effect

of the low-level endotoxin contamination of recombinant HSPA8 on sperm viability (membrane integrity), fresh Percoll-washed sperm samples from three boars at $5 \times 10^6/\text{ml}$ were incubated with pure endotoxin (Sigma–Aldrich; 200, 300 and 400 IU/ml) for 15 min at room temperature. In addition, a control sample with no protein and one with 0.5 $\mu\text{g}/\text{ml}$ HSPA8 were included for final comparison between the effects from different treatments.

Neutralization of the viability enhancing effect of HSPA8 by anti-HSPA8 antibody

The specificity of the observed effects was further evaluated by determining the capacity of an anti-HSPA8 antibody to block the effect of HSPA8 on sperm viability. For these experiments, fresh Percoll-washed spermatozoa from seven boars were pre-incubated with different concentrations (0, 0.1, 0.5, and 1 $\mu\text{g}/\text{ml}$) of anti-HSPA8 antibody (polyclonal rabbit IgG antibody; Abcam) for 30 min at room temperature. Thereafter, HSPA8 (0, 0.5 $\mu\text{g}/\text{ml}$) was co-incubated with pre-treated sperm for 15 min at room temperature, after which sperm viability was assessed.

Effect of sperm pre-incubation (sperm capacitation induction) on the viability enhancing effect of HSPA8

The capacitation status of boar sperm was assessed using the CTC staining method (Fazeli *et al.* 1999). To assess the influence of sperm capacitation status on the ability of HSPA8 to enhance sperm viability, a total of six boar semen samples were incubated in TALP at 39 °C (capacitating condition) for 0, 1, 2, 4, and 6 h. At each time point, HSPA8 (0 and 0.5 $\mu\text{g}/\text{ml}$) was added and samples were incubated for an additional 15 min at room temperature. The viability and capacitation status (minimum of 200 live spermatozoa per slide three replicates per sample) were then evaluated using an epifluorescence microscope.

Effect of HSPA8 on sperm mitochondrial activity

Fresh Percoll-washed sperm samples from nine boars were incubated with HSPA8 (0 and 0.5 $\mu\text{g}/\text{ml}$) and α -tubulin (0.5 $\mu\text{g}/\text{ml}$), as control protein for 15 min at room temperature. Mitochondrial activity was assessed by determining the electrochemical gradient across the mitochondrial inner membrane using a cationic, lipophilic dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide, JC-1; Sigma–Aldrich). A minimum of 200 live spermatozoa per slide (three replicates for each sample) with high or low mitochondrial activity were evaluated.

Effect of HSPA8 on sperm-OEC binding

Fresh Percoll-washed semen samples from seven boars were diluted in TALP and incubated with HSPA8 (0 and 0.5 $\mu\text{g}/\text{ml}$) for 15 min at room temperature. The samples were washed briefly by centrifugation (200 *g* for 10 min) and the pellet was resuspended in TALP. The sperm–oviductal epithelial cell binding assay was performed according to the method described in the supplementary information. The number of spermatozoa attached to 100 oviductal epithelial cells was

counted by light microscopy in three replicates. The binding assay was replicated in 7 different experimental days.

Effect of HSPA8 on IVF capacity

Briefly, aliquots of pooled semen samples ($10^7/\text{ml}$) from boars with proven fertility and high motility in mTBM medium were incubated with HSPA8 (0, 0.5, 1, 10, and 20 $\mu\text{g}/\text{ml}$) or 0.5 $\mu\text{g}/\text{ml}$ HSPA1A for 15 min at room temperature.

Groups of 50 matured denuded oocytes were placed in 50 μl droplets of fertilization medium in four-well plate dishes under mineral oil at 39 °C in 5% v/v CO_2 in air. After 30 min, fresh boar spermatozoa that had been pre-incubated with HSPA8, were diluted to the appropriate concentration for insemination and 50 μl of diluted spermatozoa were added to oocytes in IVF droplets to give a final concentration of 25 000 spermatozoa (sperm:oocyte ratio, 500:1).

After 5-h incubation, oocytes from each group were transferred to 500 μl drops embryo culture medium. Sixteen hours after culture, half of the oocytes were fixed and stained for the assessment of fertilization parameters including penetration, monospermy, and efficiency rates.

Oocytes with one or more sperm heads and/or male pronuclei and the sperm tails were counted as penetrated. Penetration percentage was calculated as percentage of penetrated oocytes/total inseminated oocyte, monospermy was calculated as percentage of monospermic oocytes/total penetrated oocyte, and efficiency of fertilization was calculated as percentage of monospermic oocytes/total inseminated oocyte.

The rest of the oocytes were continued in culture for the assessment of cleavage and blastocyst formation rates (early embryonic development) on the 2nd and 7th days after IVF respectively. Cleavage rate was calculated as the percentage of embryos at two or four cells/total cultivated zygotes. Blastocyst formation rate represented the percentage of blastocysts/total cultivated zygotes. Cells per blastocysts were counted after fixing and staining with fluorescent Hoechst-33342 nuclear stain. IVF experiments were repeated in six replicates on 6 different days. Pooled data from individual replicates for oocyte penetration, monospermy, cleavage, and blastocyst formation rate as well as the number of cells/blastocyst were analyzed.

Effect of HSPA8 on sperm membrane fluidity and its specificity

Percoll-washed spermatozoa in Tyrodes solution were loaded with the lipid probe ODAF and incubated for 15 min with HSPA8 (0 and 0.5 $\mu\text{g}/\text{ml}$) or HSPA1A (0.5 $\mu\text{g}/\text{ml}$) and their membrane fluidity was assessed by FRAP. FRAP analysis was performed in three replicates (30 spermatozoa/replicate) on acrosomal and postacrosomal domains of live stained spermatozoa.

Neutralization of HSPA8 effect on sperm membrane lateral mobility by anti-HSPA8 antibody

We explored if blocking HSPA8 with a polyclonal anti-HSPA8 antibody would inhibit the protein's effect on sperm membrane lateral mobility. For this, fresh Percoll-washed spermatozoa from four boars loaded with ODAF were exposed to

Figure 1 Analysis of purified overexpressed HSPA8 in an *Escherichia coli*-based protein expression system. (A) SDS-PAGE of HSPA8 overexpressed crude *E. coli* lysate stained with Coomassie blue stain, (B) dialyzed elution of the same lysate showing enrichment of a protein around 70 kDa MW, and (C) western blot using anti-HSPA8 as primary antibody. Arrow indicates a protein band at MW 73 kDa. Western blot analysis showed this band to react with anti-HSPA8 antibody. MW, molecular weight.

anti-HSPA8 antibody (0, 0.5, and 1 µg/ml) for 30 min. Thereafter, pre-treated sperm samples were co-incubated with HSPA8 (0 and 0.5 µg/ml) for 15 min at room temperature. FRAP analysis was performed and a minimum number of 30 spermatozoa were analyzed in each sample.

Effect of sperm pre-incubation (capacitation induction) on membrane lipid lateral mobility alterations induced by short exposure to HSPA8

To test the effect of sperm capacitation on the ability of HSPA8 to alter membrane fluidity, fresh semen samples and samples pre-incubated in TALP medium at 39°C for 6 h in capacitating conditions (six different boars) were loaded with ODAF and incubated with HSPA8 (0, 0.5 µg/ml) in Tyrodes solution for 15 min at room temperature. FRAP analysis was performed and a minimum of 30 spermatozoa were analyzed in each sample.

Effect of membrane cholesterol depletion/repletion on HSPA8 viability and membrane fluidity enhancing ability

Membrane cholesterol was removed from Percoll-washed semen sample using MβCD and membrane cholesterol was measured. Spermatozoa were then treated with HSPA8 (0 and 0.5 µg/ml) for 15 min at room temperature and viability and membrane fluidity were determined.

Alternatively, cholesterol was repleted to cholesterol-depleted spermatozoa with MβCD-loaded cholesterol (CLC) after exposure to HSPA8 (0 and 0.5 µg/ml) for 15 min at room temperature. Then viability and membrane fluidity were determined.

Statistical analysis

Statistical analysis was performed using factorial ANOVA (Statistica software, version 7 (www.StatSoft.com, 1984–2004)).

Fisher least significant difference test was used to compare the effect of treatments within experimental designs. *T*-tests were used for two independent samples and the results were indicated. The results were expressed as mean ± S.E.M., and *P* values <0.05 were considered as representing statistically significant differences.

Results

In vitro expression of recombinant bovine HSPA8

The bovine *HSPA8* gene was successfully cloned into an *E. coli* expression system and produced in small (50 ml) and larger (500 ml) scale bioreactors. On an average, 14 mg pure HSPA8 protein was produced from 500 ml cultures (Fig. 1A and 1B). Western blot analysis with a HRP-conjugated anti-His (c-term) antibody confirmed the presence of His-tagged protein and an anti-HSPA8 antibody confirmed the identity of the recombinant product (Fig. 1C). The lack of reactivity with an anti-HSPA1A antibody confirmed the identity and purity of the recombinant protein.

HSPA8 enhances sperm viability in a specific manner

Both short-term (15 min) and long-term (24 and 48 h) co-incubation of fresh sperm with HSPA8 significantly enhanced sperm viability ($P \leq 0.0001$), regardless of the HSPA8 concentration. However, this effect was most pronounced at 15 min and 24 h for 0.5 µg/ml and at 48 h for 1 µg/ml (Fig. 2).

Figure 2 Effects of HSPA8 on boar sperm viability. Viability% (determined by SYBR-14/propidium iodide staining) of boar spermatozoa incubated for 15 min at room temperature, 24 and 48 h at 39 °C in the presence of different concentrations of HSPA8. Significant beneficial effects of protein treatments on sperm viability were compared to controls at specific time intervals. Different letters denote significant ($P < 0.05$) differences within specific time points. $n = 10$, means ± S.E.M.

Figure 3 The specificity of HSPA8-mediated effects and the additive effect of HSPA1A on sperm viability. (A) Sperm viability% (determined by SYBR-14/propidium iodide staining) of boar spermatozoa after 15-min exposure to 0 (control), 0.5 µg/ml HSPA8, 0.5 µg/ml α -tubulin, and 0.1, 0.5, and 1 µg/ml HSPA1A, (B) sperm viability% of boar spermatozoa after 15-min exposure to 0 (control), 0.5 µg/ml HSPA8, 0.5 µg/ml anti-HSPA8 antibody, and combination of 0.5 µg/ml HSPA8 with 0.1, 0.5, and 1 µg/ml anti-HSPA8 antibody, and (C) sperm viability% of boar spermatozoa after 15-min exposure to 0 (control), 0.5 µg/ml HSPA8, and HSPA1A-HSPA8 combinations (0.1–0.1, 0.5–0.5, 0.5–0.1, and 1–0.5 µg/ml), * $P < 0.05$, $n = 9$, means $\pm$ S.E.M.

Co-incubation of boar spermatozoa with HSPA8 for 15 min was sufficient to enhance sperm viability when evaluated using two different dye exclusion methods: calcein-AM/ethidium homodimer (viability: $65.3\% \pm 1$ vs $78.18\% \pm 1.5$ for control vs HSPA8-treated samples respectively, mean $\pm$ S.E.M., $n = 10$, $P < 0.005$) and SYBR-14/propidium iodide ($67.1\% \pm 1$ vs $78.7\% \pm 2$ respectively, means $\pm$ S.E.M., $n = 10$, $P < 0.005$).

Low contaminating background levels of endotoxin had no detrimental effect on sperm viability (membrane integrity) during 15-min incubation (69 ± 5 vs 73.5 ± 5.5 vs 68 ± 4 vs 69 ± 6.5 , control vs 200 vs 300 vs 400 IU/ml endotoxin-treated samples respectively, mean $\pm$ S.E.M., $n = 3$), whereas HSPA8 significantly increased sperm

viability ($69\% \pm 5$ vs $81\% \pm 6$, control vs HSPA8-treated samples respectively, means $\pm$ S.E.M., $n = 3$, $P < 0.01$).

The specificity of the viability-enhancing effects of HSPA8 (0.5 µg/ml) was evaluated by comparing the effect

Figure 4 Effect of sperm pre-incubation (capacitation induction) on the ability of HSPA8 to enhance boar sperm viability. (A) Viability% (determined by SYBR-14/propidium iodide staining), (B) capacitation% (assessed by chlortetracycline assay of boar spermatozoa) after incubation at 0, 1, 2, 4, and 6 h at 39 °C in capacitation condition followed by 15-min exposure to 0.5 µg/ml HSPA8 at room temperature. (A) *Significant ($P < 0.05$) differences within specific time points, (B) *significant ($P < 0.05$) differences between different incubation time points and control (0 h), and (C) percentage change in viability and capacitation status of boar spermatozoa after incubation at 1, 2, 4, and 6 h at 39 °C in capacitation condition followed by 15-min exposure to 0.5 µg/ml HSPA8 at room temperature. $n = 6$, means $\pm$ S.E.M., $r = 0.8$.

Table 1 Boar spermatozoa were treated with and without HSPA8 (0.1, 0.5, 1, 10, and 20 µg/ml) or HSPA1A (0.5 µg/ml) 15 min prior to insemination at room temperature. Sixteen hours post-insemination, half the oocytes were fixed and stained for assessment of IVF parameters (penetration, monospermy, and efficiency rates).

Experimental group	Total	No. of matured oocytes	Penetrated (%)	Monospermy (%)	Efficiency (%)
Control	317	287	195 (68.3±3.9%)	68 (36.4±5.5%)	68 (21.5±2.8%)
HSPA8, 0.5 µg/ml	319	286	213 (74.9±3.9%)	119 (56.8±5.4%)*	119 (37.2±2.8%)*
HSPA8, 1 µg/ml	346	301	243 (81.4±3.9%)*	146 (60.2±5.3%)*	146 (42.1±2.7%)*
HSPA8, 10 µg/ml	335	297	220 (74.5±3.9%)	106 (49.1±5.4%)	106 (31.6±2.7%)
HSPA8, 20 µg/ml	333	295	202 (69.8±3.9%)	98 (49.0±5.5%)	98 (29.4±2.7%)
HSPA1A, 0.5 µg/ml	332	301	219 (73.2±3.9%)	86 (39.2±5.4%)	86 (25.9±2.7%)

* $P < 0.05$, $n = 6$ replicates, means $\pm$ S.E.M.

with that of HSPA1A and α -tubulin, and also determining whether the effect could be blocked using an anti-HSPA8 antibody. Although exposure of spermatozoa to HSPA8 significantly enhanced sperm viability, neither HSPA1A nor α -tubulin had similar effects on sperm survival (Fig. 3A). The anti-HSPA8 antibody blocked the viability enhancing effect of HSPA8 in a concentration-dependent manner (Fig. 3B). Interestingly, the presence of HSPA1A appeared to block the positive effects of HSPA8 on sperm viability (Fig. 3C).

Sperm capacitation negates the viability-enhancing effects of HSPA8

Contrary to the above, the addition of HSPA8 (0.5 µg/ml) to spermatozoa pre-incubated in a capacitating medium had no significant effect on their viability or capacitation status, as assessed by their CTC staining characteristics (Fig. 4A and B). However, the proportion of spermatozoa responding to the viability-enhancing effect of HSPA8 decreased as the incubation progressed and the rate of capacitation increased. There was a strong negative correlation between the proportion of capacitated spermatozoa and the increase in the proportion of live sperm in response to HSPA8 treatment ($R = -0.81$, $P = 0.0038$, Fig. 4C).

HSPA8 reduces sperm mitochondrial activity

JC-1 differentially labels mitochondria with high- and low-membrane electrical activity red or green

respectively. HSPA8 reduced mitochondrial activity of fresh spermatozoa (proportion of spermatozoa with high mitochondrial activity after 15-min treatment $72.73\% \pm 1.5$ vs $64.83\% \pm 1.5$ for control and HSPA8-treated sperm samples respectively, $P \leq 0.01$), whereas α -tubulin had no effect ($70.73\% \pm 1.5$ vs $73\% \pm 3$ respectively). Data are presented as means $\pm$ S.E.M., $n = 9$.

HSPA8 increases sperm-OEC binding

Exposure of spermatozoa to HSPA8 for 15 min significantly increased the mean number of spermatozoa that attached to epithelial cells ($9\% \pm 3$ vs $14\% \pm 1.5$ spermatozoa/epithelial cell for control vs HSPA8-treated sperm samples respectively ($P \leq 0.05$), whereas α -tubulin had no effect on sperm-OEC binding $9\% \pm 3$ vs $7.5\% \pm 1.15$ spermatozoa/epithelial cell respectively. Data are presented as means $\pm$ S.E.M., $n = 7$.

HSPA8 improves the IVF capacity of sperm

A total of 4027 oocytes in six replicates were inseminated, from which 1982 oocytes were assessed for parameters of IVF and the rest (2045) for *in vitro* culture parameters. As indicated in Table 1, a 15-min incubation of semen with 1 µg/ml HSPA8 significantly increased penetration rate ($68.3\% \pm 3.9$ vs $81.4\% \pm 3.9$, for control and HSPA8-treated samples respectively, $P < 0.004$). None of the other concentrations tested had any effect. In addition, monospermy significantly increased when spermatozoa were pre-treated with

Table 2 Boar spermatozoa were treated with and without HSPA8 (0.1, 0.5, 1, 10, and 20 µg/ml) or HSPA1A (0.5 µg/ml) 15 min prior to insemination at room temperature. The rest of the oocytes were continued in culture for assessment of *in vitro* culture parameters (cleavage, blastocyst formation rate, and mean cell number/blastocyst) on the 2nd and 7th days after IVF respectively.

Experimental group	Total	Cleaved (%)	Blastocyst (%)	Mean number of cell/blastocyst
HSPA8, 0 µg/ml	326	163 (49.9±3.5%)	(19.5±4.5%)	33.19±2.3
HSPA8, 0.5 µg/ml	351	174 (49.3±3.4%)	(23.7±4.4%)	38.03±2.1
HSPA8, 1 µg/ml	345	178 (51.4±3.5%)	(21.0±4.5%)	34.66±2.2
HSPA8, 10 µg/ml	349	154 (43.9±3.5%)	(20.3±4.4%)	34.67±2.2
HSPA8, 20 µg/ml	341	158 (46.1±3.5%)	(16.5±4.5%)	37.98±2.4
HSPA1A, 0.5 µg/ml	333	156 (46.6±3.5%)	(21.4±4.5%)	34.37±2.2

0.5 $\mu\text{g/ml}$ ($36.4\% \pm 5.5$ vs $56.8\% \pm 5.4$, $P < 0.0001$) and 1 $\mu\text{g/ml}$ ($36.4\% \pm 5.5$ vs $60.2\% \pm 5.3$, $P < 0.0001$) of HSPA8. The efficiency rate was accordingly higher after incubating sperm with 0.5 and 1 $\mu\text{g/ml}$ HSPA8.

There were no significant differences in cleavage rate or blastocyst formation rate between the groups, and HSPA8 had no apparent effect on the quality of the final embryo, as evaluated by the mean number of nuclei/blastocyst. HSPA1A had no effect on any of the measured parameters (Table 2).

HSPA8 increases lipid diffusion on the sperm head

Exposure of spermatozoa to HSPA8 significantly and rapidly increased diffusion co-efficient (D -values $10^{-9} \text{ cm}^2/\text{s}$) and recovery% (R%) of sperm plasma membrane lipids in the acrosomal and postacrosomal regions of the sperm head. HSPA1A had no effect on any of the sperm membrane fluidity parameters in the two regions (Fig. 5). Moreover, anti-HSPA8 antibody blocked the enhancing effect of HSPA8 on boar sperm membrane fluidity on both regions (Fig. 6).

Capacitation counteracts the membrane fluidity enhancing effect of HSPA8

Incubation of spermatozoa in capacitating conditions for 6 h significantly increased the percentage of capacitated spermatozoa from $7\% \pm 2.5$ at 0 h to $67\% \pm 5.5$ at 6 h, as assessed by the CTC staining assay. Capacitation *per se* also caused a significant increase in D -values for ODAF over the acrosome and post-acrosome relative to non-capacitated spermatozoa (Fig. 7A, B, C, and D). However, acute exposure of capacitated spermatozoa to 0.5 $\mu\text{g/ml}$ HSPA8 had no additional effect on any of the measured parameters in any of the sperm head regions (Fig. 7).

Reduction of sperm membrane cholesterol attenuates HSPA8-induced viability and membrane fluidity enhancing effects

Incubation of fresh sperm suspensions for 30 min with 0–8 mM M β CD progressively reduced their cholesterol content, with a strong inverse linear correlation between them ($r^2 = -0.99$, means $\pm$ S.E.M., $n = 5$, $P = 0.0003$; Fig. 8A). With 4 mM M β CD, cholesterol levels were estimated at $0.36 \mu\text{g} \pm 0.16/10^6$ cells relative to $0.48 \mu\text{g} \pm 0.11/10^6$ cells in control samples. In the presence of 8 mM M β CD, cholesterol was further reduced to $0.22 \mu\text{g} \pm 0.11/10^6$ cells, i.e. 56% of controls. No change in sperm viability was detected between 1 and 4 mM M β CD (Fig. 8B), but at 8 mM M β CD viability decreased from $68\% \pm 4.5$ to $53\% \pm 3$ (control vs M β CD treatment, means $\pm$ S.E.M., $n = 5$, $P = 0.003$). Thus, 4 mM M β CD lowered sperm cholesterol by 25% without

Figure 5 Effect of HSPA8 and its specificity on sperm membrane fluidity assessed by fluorescence recover after photobleaching technique. (A) Acrosome D -values ($10^{-9} \text{ cm}^2/\text{s}$), (B) postacrosome D -value, (C) acrosome recovery%, and (D) postacrosome recovery% of fresh boar spermatozoa (30 spermatozoa/sample) were analyzed after 15-min exposure to 0.5 $\mu\text{g/ml}$ HSPA8 or HSPA1A at room temperature. [†] $P < 0.05$, $n = 3$, means $\pm$ S.E.M.

Figure 6 Neutralizing effect of anti-HSPA8 antibody on HSPA8 ability to enhance membrane fluidity assessed by fluorescence recovery after photobleaching. (A) Acrosome *D*-values (10⁻⁹ cm²/s), (B) postacrosome *D*-values (10⁻⁹ cm²/s), (C) acrosome recovery%, and (D) postacrosome recovery% of fresh boar spermatozoa (30 spermatozoa/sample) in response to 0.5 µg/ml HSPA8 after pre-treatment with 0.1, 0.5, and 1 µg/ml anti-HSPA8 antibody at room temperature. **P* < 0.05, *n* = 4, means ± s.e.m.

adversely affecting their viability under the conditions employed.

In the presence of 1–4 mM MβCD, diffusion of ODAF on the sperm acrosome increased progressively from 31 to 47 × 10⁻⁹ cm²/s (*P* < 0.005), but fell sharply to 15 × 10⁻⁹ cm²/s in the presence of 8 mM MβCD (Fig. 8C). Subsequent exposure of these sperm to 0.5 µg/ml of HSPA8 attenuated the increase in ODAF diffusion, with the effect being much reduced at 8 mM MβCD. On the postacrosome, ODAF diffusion increased only at the lower concentrations of MβCD (1–2 mM), decreasing substantially at 4–8 mM MβCD. As observed on the acrosome, the addition of 0.5 µM HSPA8 increased ODAF diffusion only at the lower concentrations of MβCD (Fig. 8D).

Repletion of membrane cholesterol restored HSPA8 function on sperm membrane

Cholesterol-depleted spermatozoa did not respond to HSPA8 treatment in the same way as controls in terms of viability and membrane fluidity enhancement. However, repletion of membrane cholesterol restored the membrane responsiveness to HSPA8 effects on both viability and membrane fluidity in the sperm acrosome and postacrosomal membrane (Fig. 8E, F, and G).

Discussion

HSPA8 had an immediate effect on improving sperm viability, enhancing sperm membrane fluidity, decreasing sperm mitochondrial activity, increasing the ability of spermatozoa to bind to oviductal cells, and increasing the rate of *in vitro* monospermic fertilization. These were manifested in spermatozoa just after only 15-min (or shorter) exposure to HSPA8.

Long-term beneficial effects of HSPA8, including an improvement the survival of bull, boar, and ram sperm (24 or 48 h) are well documented (Elliott *et al.* 2009, Lloyd *et al.* 2009). Other HSPs such as HSPD (HSP60), HSPA1A, and a combination of HSPA1A/HSPA8 are also known to prolong the survival of different cell types such as sensory neuronal cells (Houenou *et al.* 1996) and vascular endothelial cells (Johnson *et al.* 1990), after extended periods of co-culture with HSPs. However, the current investigation has extended the known capacity of HSPA8 to prevent membrane damage to also include a membrane repair mechanism. A consistent and significant increase of 10–15% in the proportion of live (membrane intact) spermatozoa after brief exposure of spermatozoa to exogenous HSPA8 implied that HSPA8 is rapidly restores membrane integrity in damaged spermatozoa. Spermatozoa repaired by short exposure to HSPA8 are either morbid or undergoing the process of cell death. Hence, HSPA8 seems to have reversed the process that eventually will lead to their destruction and has restored membrane integrity in these cells.

Figure 7 Effect of sperm pre-incubation (capacitation induction) on HSPA8 ability to enhance membrane fluidity assessed by fluorescence recovery after photobleaching technique. (A) Acrosome D -values ($10^{-9} \text{ cm}^2/\text{s}$), (B) postacrosome D -values ($10^{-9} \text{ cm}^2/\text{s}$), (C) acrosome recovery%, and (D) postacrosome recovery% of boar spermatozoa (30 spermatozoa/sample) at 0 and 6 h pre-incubation in capacitating conditions, followed by 15 min exposure to 0 and 0.5 $\mu\text{g}/\text{ml}$ HSPA8. *Significant ($P < 0.05$) differences within specific time points, $n = 4$, means $\pm$ S.E.M., NS, not significant.

HSPA8 probably reverses sperm membrane damage and restores cell membrane integrity via interactions with, and alterations in, membrane properties. A number of studies have reported on the ability of different HSPs to enhance viability via interactions with cellular membranes in different cell types (Johnson *et al.* 1990, Johnson & Tytell 1993, Boilard *et al.* 2004, Elliott *et al.* 2009). It is known that HSPs regulate membrane stability and fluidity (Chen *et al.* 2005, Horvath *et al.* 2008) and that HSPs are involved in stabilizing cellular and organellar membranes in stressful conditions (Carratu *et al.* 1996, Vigh *et al.* 1998, 2007). Interaction of HSP17 with *Synechocystis* membrane lipids positively influences membrane integrity and fluidity in favor of cell survival under stressful conditions (Horvath *et al.* 1998). An *E. coli* HSP, GroEL, has also been shown to associate with and stabilize bacterial membrane during

stress (Torok *et al.* 1997). Furthermore, HSPA1A prevents cancer cell death by inhibiting lysosomal membrane permeabilization (Gyrd-Hansen *et al.* 2004, Nylandsted *et al.* 2004, Kirkegaard *et al.* 2010). Generally, it appears that HSP-cell membrane associations antagonize stress-induced disturbances of membrane domains and preserve functional and structural integrity of the biomembranes (Horvath *et al.* 1998, 2008, Vigh *et al.* 2005). Our current observations of an immediate effect of HSPA8 on spermatozoa extend HSPA8 from a protective agent to a molecule which is capable of repair of cell integrity.

In the current study, we used FRAP to measure two-dimensional lateral dynamics (fluidity) and topology of molecules within a defined area of membrane bilayer (Ladha *et al.* 1997, Wolfe *et al.* 1998). FRAP demonstrated that short exposure to HSPA8 enhanced sperm membrane lipid fluidity in the acrosomal and post-acrosomal domains. Diffusion coefficient levels and recovery values were within the range that would be expected to reflect viability and membrane intactness (Ladha *et al.* 1997, Wolfe *et al.* 1998). Potentially, this rapid increase in membrane fluidity after exposure to exogenous HSPA8 is the mechanism that is responsible for restoration of cell membrane integrity in damaged cells by HSPA8. Fluidity is a characteristic feature of membranes. Cellular viability and function is dependent on maintenance of cell membrane fluidity within physiological ranges (Horvath *et al.* 1998, 2008). A set of factors ranging from naturally occurring non-hazardous intracellular activities to deleterious environmental stressors constantly disturb the membrane fluidity equilibrium (Horvath *et al.* 1998, Horvath & Vigh 2010). These events individually or in combination can disrupt membrane stability by shifting the membrane fluidity pattern below or above the physiological range specific to each cell system. For instance, high temperatures induce hyperfluidity (Wolfe *et al.* 1998), whereas oxidation causes rigidity in cell membranes (Christova *et al.* 2004). It is therefore very possible that HSPA8 increased membrane lipid fluidity in the subpopulation of spermatozoa with reduced fluidity due to stress, cold, oxidative physical damage, or any other membrane disturbing factors.

Depletion of membrane cholesterol diminished the effect of HSPA8 on both membrane integrity and fluidity. Hence, the immediate conclusion would be that there is a biological interaction between cholesterol and HSPA8 molecules, without which the effect of HSPA8 on the sperm membrane is abolished. That idea was supported when further experiments showed that the HSPA8 effect was restored maintained after cholesterol replenishment. However, whether cholesterol molecules act as binding sites for HSPA8 or only plays a supporting role by maintaining the raft structures tightly together so that HSPA8 molecules can attach to their binding sites/receptors on the organized lipid plane has yet to

Figure 8 Effect of sperm membrane cholesterol modification on HSPA8 function on sperm membrane integrity and fluidity. (A) Cholesterol content ($\mu\text{g}/10^6$ spermatozoa) of boar sperm membrane after 30-min treatment with M β CD (0, 1, 2, 4, and 8 mM/ 10^8 spermatozoa) at room temperature analyzed by spectrophotometry. Mean $\pm$ S.E.M., $r^2 = -0.99$, (B) viability, (C) acrosome D -values ($10^{-9} \text{ cm}^2/\text{s}$), (D) postacrosome D -value of boar spermatozoa (30 spermatozoa) after depletion of membrane cholesterol by M β CD (0, 1, 2, 4, and 8 mM/ 10^8 spermatozoa) and 15-min exposure to 0.5 $\mu\text{g}/\text{ml}$ HSPA8, (E) viability%, (B–E) * represents significant difference between individual groups, $P < 0.05$, $n = 5$, mean $\pm$ S.E.M. (F) acrosome D -values ($10^{-9} \text{ cm}^2/\text{s}$), and (G) and postacrosome D -values ($10^{-9} \text{ cm}^2/\text{s}$) of boar spermatozoa ($n = 30$) after repletion of membrane cholesterol by CLC (2 mg/ 10^8 spermatozoa) to spermatozoa that were already membrane cholesterol depleted by M β CD 4 mM/ 10^8 spermatozoa and 15-min exposure to 0.5 $\mu\text{g}/\text{ml}$ HSPA8, different letters denote significant differences within specific treatments. $P < 0.05$, $n = 5$, means $\pm$ S.E.M.

be determined. The proposed concept can also explain the inability of HSPA8 to enhance the viability of capacitated spermatozoa. Capacitation involves substantial reduction in membrane cholesterol (Osheroff *et al.* 1999). It is therefore highly probable that the capacitation-induced reduction/reorganization in sperm membrane cholesterol/lipid structure compromises the interaction sites for HSPA8 to assert its effects.

As sperm capacitation is a multistep process, developing direct, simple, and straightforward assays for capacitation has been an issue of debate. All the capacitation assays available so far are indirect ones, which evaluate this complex process based on different steps and definitions. As stated previously, CTC fluorescence assay was used for assessment of sperm capacitation status in our experiments. CTC produces distinct fluorescence distribution patterns on the sperm surface depending on the capacitation and acrosomal status of sperm (Visconti *et al.* 1998). This assay was first described by Saling & Storey (1979). Some years later, the correlation between sperm capacitation status and the previously described patterns were validated (Ward & Storey 1984). The fluorescent neutral chlortetracycline is transferred across the cell membrane, complexes with intracellular free Ca^{2+} and this leads to an increase in its fluorescence intensity. Preferential binding of the CTC- Ca^{2+} complex to the hydrophobic regions of the sperm membrane results in the different fluorescence patterns described before (Visconti *et al.* 1998). The advantage of the CTC assay is that it evaluates the final stages of capacitation independently of the acrosome reaction. However, as it is assumed that fluctuations in the distribution of Ca^{2+} -CTC complexes that bind to membrane phospholipid molecules are responsible for the different patterns observed, care must be taken in interpretation of the results and assay design because any factor that affects the fluorescence absorption spectrum of the CTC or of Ca^{2+} -phospholipid complex or that quenches the fluorescence intensity of these complexes could cause potential misinterpretation in the capacitation status of the spermatozoa (Visconti *et al.* 1998).

In contrast to HSPA8, human recombinant HSPA1A had no effect on sperm viability or membrane lipid lateral diffusion in this study. These results were partly unexpected because of the wealth of literature supporting HSPA1A's role in the protection of different cell types (Beckmann *et al.* 1990, Shi & Thomas 1992, Browne *et al.* 2007). Furthermore, none of the HSPA1A and HSPA8 combinations had any effect on viability, suggesting that HSPA1A negates the positive effect that is observed with HSPA8 alone. This observation confirmed recent reports of differential and antagonizing effects of HSPA1A against HSPA8 in other cellular systems. In *Xenopus* oocytes, the induction of surface expression of epithelial sodium channels by individual HSPA1A is antagonized by co-injection of HSPA1A and HSPA8 cRNA to the cells in a concentration-dependant manner (Goldfarb *et al.* 2006). Furthermore, these two proteins have been shown to counteract the stabilization of a potassium channel subunit in human cardiomyocytes (Li *et al.* 2011). Finally, the ability to block the effects of HSPA8 on sperm viability and membrane fluidity using an anti-HSPA8 antibody confirmed the unique and specific effect of short exposure of sperm to HSPA8 in restoring sperm viability and not other HSPs such as HSPA1A.

Among all body organs, the female reproductive tract holds a unique responsibility in maintaining sperm viability and fertilizing capacity that is most vital in species where there is a long time span between sperm deposition in the tract and ovulation (Lloyd *et al.* 2009). Therefore, perpetuating reproduction necessitates consistent function and persistent expression of factors necessary for protection and maintenance of sperm survival in the female reproductive organ. When exposed to stress, many body organs express HSPA1A as a survival prolonging factor to delay their death (Schmitt *et al.* 2007, Kirkegaard *et al.* 2010). HSPA1A is the stress-induced HSP whose expression and cell protecting functions are restricted to times of stress (Schlesinger 1990, Craig *et al.* 1993). However, the female reproductive tract appears to use HSPA8, which is a constitutive HSP and is present in the cells at all times (Schlesinger 1990, Craig *et al.* 1993). Replacement of HSPA1A by HSPA8 as a viability protecting agent in the female reproductive system might be an evolutionary solution which ensures the constant presence of regulatory factors in the sperm reservoir. This, in part, can explain the inefficiency of HSPA1A in enhancing sperm viability in our system opposed to other biological systems.

In conclusion, this study demonstrated that a brief exposure of boar spermatozoa to extracellular recombinant HSPA8 significantly increased sperm viability, decreased mitochondrial activity, increased the capacity of sperm to bind OECs, and improved sperm IVF rates. Our findings suggested that exogenous HSPA8 rapidly affected spermatozoa by changing membrane fluidity parameters. Moreover, cholesterol is required for HSPA8 to exert its effects on sperm membrane.

The immediate impact of exogenous HSPA8 on sperm viability and function provides a physiological basis for the concept that HSPs secreted/released in the female reproductive tract or the other body fluids have a role as 'rapid response' extracellular cytoprotectors and modifiers of cellular function.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

N Moein-Vaziri and A Fazeli designed experiments and wrote the manuscript. N Moein-Vaziri conducted the experiments.

I Philips and S Smith produced the recombinant HSPA8. C Maside, M A Gil, J Roca and E A Martinez helped for design, conduction and statistical analysis of IVF experiments. W V Holt and A G Pockley contributed significantly to the experimental design and scientific advice.

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References

- Abe H, Sendai Y, Satoh T & Hoshi H 1995 Bovine oviduct-specific glycoprotein: a potent factor for maintenance of viability and motility of bovine spermatozoa *in vitro*. *Molecular Reproduction and Development* **42** 226–232. (doi:10.1002/mrd.1080420212)
- Abeysdeera LR & Day BN 1997 *In vitro* penetration of pig oocytes in a modified Tris-buffered medium: effect of BSA, caffeine and calcium. *Theriogenology* **48** 537–544. (doi:10.1016/S0093-691X(97)00270-7)
- Beckmann RP, Mizzen LE & Welch WJ 1990 Interaction of Hsp 70 with newly synthesized proteins: implications for protein folding and assembly. *Science* **248** 850–854. (doi:10.1126/science.2188360)
- Boilard M, Reyes-Moreno C, Lachance C, Massicotte L, Bailey JL, Sirard MA & Leclerc P 2004 Localization of the chaperone proteins GRP78 and HSP60 on the luminal surface of bovine oviduct epithelial cells and their association with spermatozoa. *Biology of Reproduction* **71** 1879–1889. (doi:10.1095/biolreprod.103.026849)
- Browne CL, Swan JB, Rankin EE, Calvert H, Griffiths S & Tytell M 2007 Extracellular heat shock protein 70 has novel functional effects on sea urchin eggs and coelomocytes. *Journal of Experimental Biology* **210** 1275–1287. (doi:10.1242/jeb.02743)
- Burdon RH 1986 Heat shock and the heat shock proteins. *Biochemical Journal* **240** 313–324.
- Carratu L, Franceschelli S, Pardini CL, Kobayashi GS, Horvath I, Vigh I & Maresca B 1996 Membrane lipid perturbation modifies the set point of the temperature of heat shock response in yeast. *PNAS* **93** 3870–3875. (doi:10.1073/pnas.93.9.3870)
- Chen S, Bawa D, Besshoh S, Gurd JW & Brown IR 2005 Association of heat shock proteins and neuronal membrane components with lipid rafts from the rat brain. *Journal of Neuroscience Research* **81** 522–529. (doi:10.1002/jnr.20575)
- Christova Y, James PS & Jones R 2004 Lipid diffusion in sperm plasma membranes exposed to peroxidative injury from oxygen free radicals. *Molecular Reproduction and Development* **68** 365–372. (doi:10.1002/mrd.20084)
- Craig EA, Gambill BD & Nelson RJ 1993 Heat shock proteins: molecular chaperones of protein biogenesis. *Microbiological Reviews* **57** 402–414.
- Elliott RM, Lloyd RE, Fazeli A, Sostaric E, Georgiou AS, Satake N, Watson PF & Holt WV 2009 Effects of HSPA8, an evolutionarily conserved oviductal protein, on boar and bull spermatozoa. *Reproduction* **137** 191–203. (doi:10.1530/REP-08-0298)
- Fazeli A, Duncan AE, Watson PF & Holt WV 1999 Sperm–oviduct interaction: induction of capacitation and preferential binding of uncapacitated spermatozoa to oviductal epithelial cells in porcine species. *Biology of Reproduction* **60** 879–886. (doi:10.1095/biolreprod60.4.879)
- Fazeli A, Elliott RM, Duncan AE, Moore A, Watson PF & Holt WV 2003 *In vitro* maintenance of boar sperm viability by a soluble fraction obtained from oviductal apical plasma membrane preparations. *Reproduction* **125** 509–517. (doi:10.1530/rep.0.1250509)
- Georgiou AS, Sostaric E, Wong CH, Snijders AP, Wright PC, Moore HD & Fazeli A 2005 Gametes alter the oviductal secretory proteome. *Molecular & Cellular Proteomics* **4** 1785–1796. (doi:10.1074/mcp.M500119-MCP200)

- Goldfarb SB, Kashlan OB, Watkins JN, Suaud L, Yan W, Kleyman TR & Rubenstein RC 2006 Differential effects of Hsc70 and Hsp70 on the intracellular trafficking and functional expression of epithelial sodium channels. *PNAS* **103** 5817–5822. (doi:10.1073/pnas.0507903103)
- Green CE, Bredl J, Holt WV, Watson PF & Fazeli A 2001 Carbohydrate mediation of boar sperm binding to oviductal epithelial cells *in vitro*. *Reproduction* **122** 305–315. (doi:10.1530/rep.0.1220305)
- Grippio AA, Way AL & Killian GJ 1995 Effect of bovine ampullary and isthmic oviductal fluid on motility, acrosome reaction and fertility of bull spermatozoa. *Journal of Reproduction and Fertility* **105** 57–64. (doi:10.1530/jrf.0.1050057)
- Guzhova I, Kislyakova K, Moskaliyova O, Fridlanskaya I, Tytell M, Cheetham M & Margulis B 2001 *In vitro* studies show that Hsp70 can be released by glia and that exogenous Hsp70 can enhance neuronal stress tolerance. *Brain Research* **914** 66–73. (doi:10.1016/S0006-8993(01)02774-3)
- Gyrd-Hansen M, Nylandsted J & Jaattela M 2004 Heat shock protein 70 promotes cancer cell viability by safeguarding lysosomal integrity. *Cell Cycle* **3** 1484–1485. (doi:10.4161/cc.3.12.1287)
- Hightower LE & Guidon PT Jr 1989 Selective release from cultured mammalian cells of heat-shock (stress) proteins that resemble gliaxon transfer proteins. *Journal of Cellular Physiology* **138** 257–266. (doi:10.1002/jcp.1041380206)
- Holt WV & Harrison RA 2002 Bicarbonate stimulation of boar sperm motility via a protein kinase A-dependent pathway: between-cell and between-ejaculate differences are not due to deficiencies in protein kinase A activation. *Journal of Andrology* **23** 557–565.
- Horvath I & Vigh L 2010 Cell biology: stability in times of stress. *Nature* **463** 436–438. (doi:10.1038/463436a)
- Horvath I, Glatz A, Varvasovszki V, Torok Z, Pali T, Balogh G, Kovacs E, Nadasdi L, Benko S, Joo F *et al.* 1998 Membrane physical state controls the signaling mechanism of the heat shock response in *Synechocystis* PCC 6803: identification of hsp17 as a “fluidity gene”. *PNAS* **95** 3513–3518. (doi:10.1073/pnas.95.7.3513)
- Horvath I, Multhoff G, Sonnleitner A & Vigh L 2008 Membrane-associated stress proteins: more than simply chaperones. *Biochimica et Biophysica Acta* **1778** 1653–1664. (doi:10.1016/j.bbame.2008.02.012)
- Houenou LJ, Li L, Lei M, Kent CR & Tytell M 1996 Exogenous heat shock cognate protein Hsc 70 prevents axotomy-induced death of spinal sensory neurons. *Cell Stress & Chaperones* **1** 161–166. (doi:10.1379/1466-1268(1996)001<0161:EHSCPH>2.3.CO;2)
- Johnson AD & Tytell M 1993 Exogenous HSP70 becomes cell associated, but not internalized, by stressed arterial smooth muscle cells. *In Vitro Cellular & Developmental Biology. Animal* **29A** 807–812. (doi:10.1007/BF02634348)
- Johnson AD, Berberian PA & Bond MG 1990 Effect of heat shock proteins on survival of isolated aortic cells from normal and atherosclerotic cynomolgus macaques. *Atherosclerosis* **84** 111–119. (doi:10.1016/0021-9150(90)90080-3)
- Kirkegaard T, Roth AG, Petersen NH, Mahalka AK, Olsen OD, Moilanen I, Zylcz A, Knudsen J, Sandhoff K, Arenz C *et al.* 2010 Hsp70 stabilizes lysosomes and reverts Niemann–Pick disease-associated lysosomal pathology. *Nature* **463** 549–553. (doi:10.1038/nature08710)
- Ladha S, James PS, Clark DC, Howes EA & Jones R 1997 Lateral mobility of plasma membrane lipids in bull spermatozoa: heterogeneity between surface domains and rigidification following cell death. *Journal of Cell Science* **110** 1041–1050.
- Li P, Ninomiya H, Kurata Y, Kato M, Miake J, Yamamoto Y, Igawa O, Nakai A, Higaki K, Toyoda F *et al.* 2011 Reciprocal control of HERG stability by Hsp70 and Hsc70 with implication for restoration of LQT2 mutant stability. *Circulation Research* **108** 458–468. (doi:10.1161/CIRCRESAHA.110.227835)
- Lloyd RE, Elliott RM, Fazeli A, Watson PF & Holt WV 2009 Effects of oviductal proteins, including heat shock 70 kDa protein 8, on survival of ram spermatozoa over 48 h *in vitro*. *Reproduction, Fertility, and Development* **21** 408–418. (doi:10.1071/RD08204)
- Moore AI, Squires EL & Graham JK 2005 Adding cholesterol to the stallion sperm plasma membrane improves cryosurvival. *Cryobiology* **51** 241–249. (doi:10.1016/j.cryobiol.2005.07.004)
- Nylandsted J, Gyrd-Hansen M, Danielewicz A, Fehrenbacher N, Lademann U, Hoyer-Hansen M, Weber E, Multhoff G, Rohde M & Jaattela M 2004 Heat shock protein 70 promotes cell survival by inhibiting lysosomal membrane permeabilization. *Journal of Experimental Medicine* **200** 425–435. (doi:10.1084/jem.20040531)
- Osheroff JE, Visconti PE, Valenzuela JP, Travis AJ, Alvarez J & Kopf GS 1999 Regulation of human sperm capacitation by a cholesterol efflux-stimulated signal transduction pathway leading to protein kinase A-mediated up-regulation of protein tyrosine phosphorylation. *Molecular Human Reproduction* **5** 1017–1026. (doi:10.1093/molehr/5.11.1017)
- Petters RM & Wells KD 1993 Culture of pig embryos. *Journal of Reproduction and Fertility. Supplement* **48** 61–73.
- Picard D, Khursheed B, Garabedian MJ, Fortin MG, Lindquist S & Yamamoto KR 1990 Reduced levels of hsp90 compromise steroid receptor action *in vivo*. *Nature* **348** 166–168. (doi:10.1038/348166a0)
- Purdy PH & Graham JK 2004 Effect of cholesterol-loaded cyclodextrin on the cryosurvival of bull sperm. *Cryobiology* **48** 36–45. (doi:10.1016/j.cryobiol.2003.12.001)
- Pursel VG & Johnson LA 1975 Freezing of boar spermatozoa: fertilizing capacity with concentrated semen and a new thawing procedure. *Journal of Animal Science* **40** 99–102.
- Saling PM & Storey BT 1979 Mouse gamete interactions during fertilization *in vitro*. Chlortetracycline as a fluorescent probe for the mouse sperm acrosome reaction. *Journal of Cell Biology* **83** 544–555. (doi:10.1083/jcb.83.3.544)
- Schlesinger MJ 1986 Heat shock proteins: the search for functions. *Journal of Cell Biology* **103** 321–325. (doi:10.1083/jcb.103.2.321)
- Schlesinger MJ 1990 Heat shock proteins. *Journal of Biological Chemistry* **265** 12111–12114.
- Schmitt E, Gehrmann M, Brunet M, Multhoff G & Garrido C 2007 Intracellular and extracellular functions of heat shock proteins: repercussions in cancer therapy. *Journal of Leukocyte Biology* **81** 15–27. (doi:10.1189/jlb.0306167)
- Shi Y & Thomas JO 1992 The transport of proteins into the nucleus requires the 70-kilodalton heat shock protein or its cytosolic cognate. *Molecular and Cellular Biology* **12** 2186–2192.
- Torok Z, Horvath I, Goloubinoff P, Kovacs E, Glatz A, Balogh G & Vigh L 1997 Evidence for a lipochaperonin: association of active protein-folding GroESL oligomers with lipids can stabilize membranes under heat shock conditions. *PNAS* **94** 2192–2197. (doi:10.1073/pnas.94.6.2192)
- Tytell M, Greenberg SG & Lasek RJ 1986 Heat shock-like protein is transferred from glia to axon. *Brain Research* **363** 161–164. (doi:10.1016/0006-8993(86)90671-2)
- Vigh L, Maresca B & Harwood JL 1998 Does the membrane's physical state control the expression of heat shock and other genes? *Trends in Biochemical Sciences* **23** 369–374. (doi:10.1016/S0968-0004(98)01279-1)
- Vigh L, Escriba PV, Sonnleitner A, Sonnleitner M, Piotto S, Maresca B, Horvath I & Harwood JL 2005 The significance of lipid composition for membrane activity: new concepts and ways of assessing function. *Progress in Lipid Research* **44** 303–344. (doi:10.1016/j.plipres.2005.08.001)
- Vigh L, Horvath I, Maresca B & Harwood JL 2007 Can the stress protein response be controlled by ‘membrane-lipid therapy’? *Trends in Biochemical Sciences* **32** 357–363. (doi:10.1016/j.tibs.2007.06.009)
- Visconti PE, Galantino-Homer H, Moore GD, Bailey JL, Ning X, Fornes M & Kopf GS 1998 The molecular basis of sperm capacitation. *Journal of Andrology* **19** 242–248.
- Ward CR & Storey BT 1984 Determination of the time course of capacitation in mouse spermatozoa using a chlortetracycline fluorescence assay. *Developmental Biology* **104** 287–296. (doi:10.1016/0012-1606(84)90084-8)
- Wolfe CA, James PS, Mackie AR, Ladha S & Jones R 1998 Regionalized lipid diffusion in the plasma membrane of mammalian spermatozoa. *Biology of Reproduction* **59** 1506–1514. (doi:10.1095/biolreprod59.6.1506)
- Young JC, Moarefi I & Hartl FU 2001 Hsp90: a specialized but essential protein-folding tool. *Journal of Cell Biology* **154** 267–273. (doi:10.1083/jcb.200104079)

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