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# Expression of Hsp60 and Grp78 in the human endometrium and oviduct, and their effect on sperm functions

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**BACKGROUND: Within the female genital tract, spermatozoa undergo a series of membranous and intracellular transformations to become competent at fertilizing the oocyte. In the bovine, previous studies have shown that two oviductal proteins, heat shock protein 60 (Hsp60) and glucose regulated protein 78 (Grp78), bind to spermatozoa and may be involved in this acquisition of fertilizing competence. METHODS: Immunohistochemical studies were performed on human endometrial and oviduct tissues to localize these two chaperones in the female genital tract. Human spermatozoa were incubated under capacitating conditions in the presence or absence of recombinant Hsp60 or Grp78. Following a 4-h incubation, the effects of these proteins were evaluated on sperm acrosomal integrity, motility, protein phosphotyrosine content and free intracellular calcium concentrations. RESULTS: Both chaperones were present in the uterus and oviduct epithelial cells and were shown to bind to human spermatozoa. Incubation with either exogenous Hsp60 or Grp78 did not affect sperm viability, motility or acrosomal integrity. Hsp60 partially prevented the increase in p81 phosphotyrosine content induced by the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine and both chaperones significantly increased the sperm intracellular calcium concentration. Moreover, the progesterone-induced increase in intracellular calcium was higher when sperm were pre-treated with either Hsp60 or Grp78. CONCLUSIONS: Our study suggests that these two proteins may affect human sperm intracellular signalling pathways and capacitation.**

*Keywords:* chaperones; intracellular calcium; phosphorylation; female reproductive tract; spermatozoa

## Introduction

Freshly ejaculated mammalian sperm must undergo a series of intracellular and membranous biochemical changes, collectively called capacitation, in order to fertilize the oocyte (Yanagimachi, 1994). These physiological modifications include increases in intracellular calcium concentration, pH, cAMP, protein tyrosine phosphorylation and membrane fluidity. In mammals, capacitation takes place in the female where sperm interact with the secretions and somatic cells located throughout the female genital tract before meeting the oocyte.

In recent years, studies on the effects of these interactions between human spermatozoa and oviduct epithelial cells (OEC), as well as their secretions, have been conducted with divergent results. Positive effects of OEC on sperm motility are generally observed, but there is much discrepancy concerning their effect observed on capacitation-associated changes. It has been reported that co-culture of human spermatozoa with OEC apical membranes enhances sperm motility and delays capacitation (Murray and Smith, 1997). Furthermore, a recent study suggested that oviductal secretions contribute to maintain sperm motility and viability, and prevent premature response to acrosome reaction inducers (Quintero *et al.*, 2005). On the other hand, incubation of spermatozoa with OEC has been shown to enhance sperm motility and hyperactivation and induce capacitation (Barratt and Cooke, 1991; Kervancioglu *et al.*, 1994,2000; Yeung *et al.*, 1994). Studies in many species have demonstrated that mammalian spermatozoa interact with and attach to OEC during their transit along the female reproductive tract (Ellington *et al.*, 1993,1999a,b; Gualtieri *et al.*, 2005). Moreover, not only do they bind to OEC, but this binding occurs selectively for higher-quality spermatozoa (Ellington *et al.*, 1999a). It has been suggested that the binding of spermatozoa to epithelium is due to interactions between cell-adhesion molecules on the cell surfaces of both spermatozoa and OEC that lead to the formation of a sperm reservoir in many mammalian species



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(Suarez, 2002). The presence of a sperm reservoir in human is not as clearly established as in other mammalian species even though some evidence support this hypothesis (Baillie *et al.*, 1997).

At the present time, few specific molecules which are secreted by or expressed at the surface of endometrial and/or oviductal epithelial cells have been shown to affect sperm signalling pathways generally associated with *in vitro* sperm capacitation. A recent study confirmed that human endometrial cell secretions induce sperm capacitation and clearly demonstrated that interleukin-6 (IL-6), which is among the factors secreted by these cells, induces capacitation by increasing tyrosine phosphorylation and the $Ca^{2+}$-ionophore-induced acrosome reaction rate (Laflamme *et al.*, 2005). Our group has identified two proteins present in the apical membrane of OEC, heat shock protein 60 (Hsp60) and glucose regulated protein 78 (Grp78), also known as BiP (immunoglobulin binding protein), that associate tightly with bull spermatozoa when incubated with OEC apical plasma membrane preparations (Boilard *et al.*, 2004). Preliminary studies suggested that incubation of cryopreserved bovine sperm with the commercial recombinant proteins Hsp60 or Grp78 improves their viability, motility and acrosome integrity (M. Boilard, unpublished data). As the previous observations demonstrated the ability of non-homologous human Hsp60 and hamster Grp78 gene products to sustain bovine sperm parameters, a similar activity could exist in human as well. This is emphasized by the fact that the amino acid sequences of molecular chaperone proteins are highly conserved between species. This hypothesis is also supported by the positive effects of bovine OEC on human sperm function. These cells were observed to be as effective as cultured human and macaque OEC during *in vitro* co-culture (Ellington *et al.*, 1998).

In humans, it has been demonstrated that the RNA encoding for Hsp60 is specifically expressed in the endometrium throughout the menstrual cycle (Tabibzadeh *et al.*, 1996), whereas no information on Hsp60 protein expression is found in the literature. In addition, there are no data on the expression of Grp78 in the human female reproductive tract, and the cellular localization of both Hsp60 and Grp78 proteins in the Fallopian tubes remains undefined. Therefore, it was imperative to determine whether these proteins are expressed by the epithelial cells lining the endometrium and oviducts with a cellular localization that would allow interaction with the sperm surface. Another objective of the present study was to determine the individual effect of these two molecular chaperones on human sperm functions and to investigate whether they modulate the acquisition of fertilizing ability of human spermatozoa.

## Materials and Methods

All experiments were conducted with approval from the ethics committee for research on human subjects from Laval University and the Laval University Medical Center (CHUL).

### *Immunohistochemistry on human uterus and oviduct tissues*

Uterine and oviductal tissues were obtained from the CHUL's Pathology Department without any information on the hormonal status or medical history of the patients. Tissues were fixed in 10% formaldehyde commercial solution (ACP, Montréal, QC, Canada) and included in paraffin by the technicians from the Pathology Department. Five paraffin-embedded tissues from human uterus and oviduct were cut into 6 μm sections. Sections were then deparaffinized in toluene and rehydrated in decreasing alcohol concentration solutions. To limit non-specific antibody interactions, sections were treated with 300 mM glycine and were next incubated in 3% hydrogen peroxide in methanol to quench endogenous peroxydase activity. Antigens were revealed by incubating sections in 10 mM boiling sodium citrate solution at pH 6. Sections were brought back to room temperature and blocked with phosphate-buffered saline (PBS) (1.5 mM $KH_2PO_4$, 8.1 mM $Na_2HPO_4$, 137 mM NaCl, 2.7 mM KCl, pH 7.4) containing 1% of bovine serum albumin (PBS/BSA 1%) for 1 h. Then, sections were incubated with anti-Hsp60 (Stressgen Biotechnologies, Victoria, BC, Canada) or anti-Grp78 (BD Biosciences-Pharmingen, San Diego, CA, USA) mouse monoclonal antibodies diluted to 10 and 5 μg/ml, respectively, in PBS/BSA 1% for 2 h at 37°C, washed five times in PBS-Tween 20 0.05% for 5 min and incubated for 30 min at room temperature with a biotinylated secondary antibody in combination with the avidin-biotin-peroxidase method (ABC vectastain Kit; Vector Laboratories, Burlingame, CA, USA) using 3,3′- diaminobenzidine (Sigma, St-Louis, MO, USA) as chromagen. Sections were counterstained with haematoxylin and mounted in water-based medium containing glycerol and Mowiol (Calbiochem, EMD Biosciences Inc., LaJolla, CA, USA) as preservative.

Slides were observed with a Zeiss Axioskop2 plus microscope (Toronto, ON, Canada) linked to a digital camera and images were acquired using Spot software (Diagnostics Instruments).

### *Semen preparation and sperm incubation*

Semen samples were obtained by masturbation from healthy volunteers after a minimum of 2 days of sexual abstinence. After liquefaction, the semen was layered on the top of a gradient composed of 2 ml fractions each of 20, 40 and 65% and 0.1 ml of 95% percoll and centrifuged (1000*g*, 30 min) to isolate spermatozoa from the seminal plasma. Percoll was made isotonic in HEPES-buffered saline (HBS; 25 mM HEPES, 130 mM NaCl, 4 mM KCl, 0.5 mM $MgCl_2$, 14 mM fructose, pH 7.6). Sperm cells at the 65–95% interface and within the 95% percoll fraction, which represent the highly motile population, were pooled and their concentration was determined by hematocytometer count. Sperm were incubated at $20 \times 10^6$ cells/ml for 4 h at 37°C (5% $CO_2$ and 100% humidity atmosphere) in modified Biggers–Whitten–Whittingham medium (BWW; 10 mM HEPES, 94.6 mM NaCl, 4.8 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 25.1 mM $NaHCO_3$, 5.6 mM D-glucose, 21.6 mM Na lactate, 0.25 mM Na pyruvate, 0.1 mg/ml phenol red, pH 7.4 and supplemented with 3 mg/ml fatty acid free BSA) (Biggers *et al*., 1971), to which 10 ng/ml commercial recombinant Hsp60 or Grp78 (Stressgen Biotechnologies) were added as required. In some experiments, spermatozoa were incubated with the general non-specific phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 500 μM) or with its solvent, dimethylsulfoxide (DMSO).

### *Biotinylation and binding of recombinant Hsp60 and Grp78 proteins to spermatozoa*

Protein biotinylation of commercial recombinant Hsp60 and Grp78 was performed with a commercial biotinylation kit Sulfo-NHS-LC-biotin [sulfosuccinimidyl-6-(biotin-amido) Hexanoate] (Pierce Biotechnology Inc., Rockford, IL, USA). Recombinant Hsp60 and Grp78 in PBS from the biotinylation kit (100 mM $Na_2HPO_4$,



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150 mM NaCl, pH 7.2) were incubated for 30 min at room temperature with sulfo-NHS-LC-biotin solution according to the manufacturer's instructions. Following incubation, unlinked biotin was removed and protein recovery was achieved by using a 10 kDa cut-off microcon Centrifugal Filter Unit (Millipore, Mississauga, ON, Canada). For the binding assays, sperm were incubated 1 h at 37°C in BWW supplemented with 100 ng/ml of biotinylated Hsp60 or Grp78 recombinant proteins. As controls, samples were incubated in BWW alone or in BWW containing a 10-fold excess of the appropriate non-biotinylated protein. Following incubation, a 10-fold volume of HBS was added and sperm were washed twice by centrifugation (500*g*, 5 min) to remove excess biotinylated proteins that did not interacted with the cells. Sperm proteins were then solubilized in electrophoresis sample buffer [2% sodium dodecyl sulphate (SDS), 10% glycerol, 50 mM dithiothreitol, 62.5 mM Tris–HCl, pH 6.8] and denatured at 100°C for 5 min. Sperm proteins were resolved by SDS-PAGE (Laemmli, 1970) and transferred onto nitrocellulose membrane (Towbin *et al.*, 1979). Non-specific binding sites on the membrane were blocked with 5% (w/v) skim milk in Tris-buffered saline containing Tween 20 (TBS-T: 150 mM NaCl, 20 mM Tris–HCl, 0.1% Tween 20, pH 7.4). The presence of biotinylated proteins on the membrane was detected using streptavidin conjugated to horseradish peroxidase (streptavidin-HRP; Jackson ImmunoResearch, WestGrove, PA, USA) for 1 h at room temperature. After five washes in TBS-T, positive bands were detected using an enhanced chemiluminescence kit (ECL; GE Healthcare Bio-Sciences Inc., Baie d'Urfé, QC, Canada) according to the manufacturer's instructions. As an alternative to biotinylated Grp78, a recombinant bovine Grp78 protein containing a N-terminal histidine tag was produced in our laboratory using the pET-16b prokaryote expression vector (Novagen Inc., Madison, WI, USA). Detection of the His-tagged Grp78 bound to sperm was done using a monoclonal anti-His tag antibody (Amersham Biosciences, Buckinghamshire, UK) and revealed with a goat anti-mouse secondary antibody conjugated to HRP (GAM-HRP; Jackson ImmunoResearch).

### Acrosome reaction assays

At the end of incubation, sperm samples were divided into two aliquots; both were washed with 1 ml PBS, centrifuged (500*g*, 5 min) and resuspended in BWW. Calcium ionophore A23 187 (Sigma) was added (10 μM final) to one of the two aliquots to induce the acrosome reaction while the other served as a control for spontaneous acrosome reactions. Both tubes were incubated for 1 h (37°C, 5% $CO_2$, 100% humidity), after which spermatozoa were washed with PBS, centrifuged (500*g*, 5 min), resuspended in PBS, smeared on slides, air-dried and fixed-permeabilized in absolute methanol for 30 min at −20°C. The acrosomal status was next evaluated according to the fluorescent pattern observed upon binding to the *Pisum sativum* agglutinin conjugated to fluorescein isothiocyanate (Sigma) (Cross *et al.*, 1986). Coverslips were mounted on slides with 90% glycerol containing 1.5% (w/v) 1,4-diazobicyclo-(2,2,2)-octane as an anti-bleaching agent and observed under UV illumination. A minimum of 200 cells were scored for each treatment.

### Evaluation of sperm motility parameters

The effects of recombinant Hsp60 or Grp78 proteins on sperm motility were assessed using a Computer-Assisted Semen Analyzer (CEROS version 12; Hamilton-Thorn Research Inc., Beverly, MA, USA). Human spermatozoa were prepared and incubated for 4 h (37°C, 5% $CO_2$, 100% humidity). All motility parameters were evaluated for a minimum of 500 spermatozoa per condition using 20 μm Microcell slides with the current setting: frame rate = 60 Hz, number of frames acquired = 30, minimum cell size = 3 pixels (setting parameters were based on CEROS technical guide version 12).

### Evaluation of protein tyrosine phosphorylation

At the end of the 4-h incubation, spermatozoa were washed with 1 ml PBS and centrifuged (500*g*, 5 min). Sperm proteins were solubilized in sample buffer, processed for electrophoresis and transferred onto nitrocellulose membrane as described earlier. Non-specific sites on the membrane were blocked with 5% (w/v) skimmed milk in TBS-T. The membrane was next incubated with a monoclonal anti-phosphotyrosine antibody (clone 4G10; Upstate Biotechnology Inc., Lake Placid, NY, USA) for 1 h at room temperature. Following several washes, the membrane was incubated with a GAM-HRP for 1 h. After five washes in TBS-T, positive immunoreactive bands were detected using the ECL kit according to the manufacturer's instructions.

To ensure that equal amount of proteins were loaded in each well of the gel, the same membrane was reprobed with a monoclonal anti-α-tubulin antibody (Sigma) using the same procedure. The intensity of the reactive bands was analysed by densitometric scanning and the ratios of phosphotyrosine/α-tubulin calculated.

### Evaluation of intracellular free calcium concentration

Percoll-washed spermatozoa were diluted to $25 \times 10^6$ cells/ml in a calcium-free BWW medium containing 2.5 μM of INDO-1 acetoxymethylester (INDO-1 AM; Molecular Probes, Eugene, OR, USA) and 0.00625% (w/v) Pluronic F-127. Following 30 min incubation in the dark at room temperature, sperm were washed with 10 ml calcium-free BWW and centrifuged (500*g*, 10 min) to remove any unincorporated $Ca^{2+}$ probe. Under these conditions, intracellular compartmentalization of the $Ca^{2+}$ probe, INDO-1 AM, was limited (Dorval *et al.*, 2002). Sperm were then resuspended in complete BWW medium, with or without 10 ng/ml of recombinant protein Hsp60 or Grp78, to a concentration of $50 \times 10^6$ cells/ml. Just before flow cytometry analyses, sperm suspensions were diluted in pre-equilibrated BWW containing propidium iodide (PI; 1.5 μg/ml) to a concentration of $1 \times 10^6$ cells/ml. Two aliquots were prepared for each sample; the basal $Ca^{2+}$ level was assessed for 1 min, at the end of which progesterone (10 μM final) was added to the first aliquot and the $Ca^{2+}$ levels were evaluated for the following 4 min. Thapsigargin (5 μM final) was added to the second tube and the intracellular $Ca^{2+}$ levels were evaluated identically. Fluorescence measurements were performed using an Epics Elite ESP flow cytometer (Beckman Coulter, Miami, FL, USA) at the beginning and after 4 h incubation at 37°C. A minimum of 10 000 sperm were analysed for each treatment.

The flow cytometer measured the $Ca^{2+}$ level by standardizing the blue emission value and then calculating the violet relative emission (VRE) for each individual sperm. Analysis of the flow cytometry results were performed using the shareware WinMDI 2.8 (http://facs.scripps.edu). The violet ($Ca^{2+}$-bound)/blue ($Ca^{2+}$-unbound) INDO-1 emission ratios were plotted versus time. First, the agglutinated sperm population and cellular debris were excluded from the analysis by using the size scatter. Damaged or dead cells that had incorporated PI were excluded. The last gating served to exclude the sperm population consisting of $[Ca^{2+}]_i$-overwhelmed sperm. Therefore, the $[Ca^{2+}]_i$ level was evaluated only for a limited sperm population that excluded agglutinated, damaged and $[Ca^{2+}]_i$-overwhelmed sperm as previously described (Boilard *et al.*, 2002).

### Statistical analysis

The data were expressed as mean ± SEM for acrosome reaction, motility parameters, tyrosine phosphorylation and calcium analyses.



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Differences among treatments were analysed by Tukey's tests after ANOVA with repeated measures.

## Results

### *Localization of Hsp60 and Grp78 in the female reproductive tract*

Immunochemistry studies demonstrated that Hsp60 and Grp78 proteins are expressed in human uterus and Fallopian tube. In the endometrium, glandular and luminal epithelial cells displayed a stronger expression of Grp78 compared with Hsp60 (Fig. 1). Unlike Hsp60, Grp78 was expressed in stromal cells as well as in epithelial cells. These two chaperones were also expressed by Fallopian tube cells (Fig. 2). Grp78 was abundantly present in all epithelial cells and showed a cytoplasmic localization on the basal and apical sides of epithelial ciliated and non-ciliated cells. Moreover, Grp78 was detected in shed nuclei and emergent vesicles coming from oviduct epithelial non-ciliated cells (Fig. 2F). As in the uterus, Grp78 was expressed by oviduct stromal cells. On the other hand, Hsp60 expression was restricted to OEC. Curiously, these cells were not uniformly labelled but rather displayed an alternation between labelled and unlabelled groups of cells of varying size. The expression pattern of either Hsp60 or Grp78 was similar in both the isthmus and ampulla sections of the Fallopian tubes.

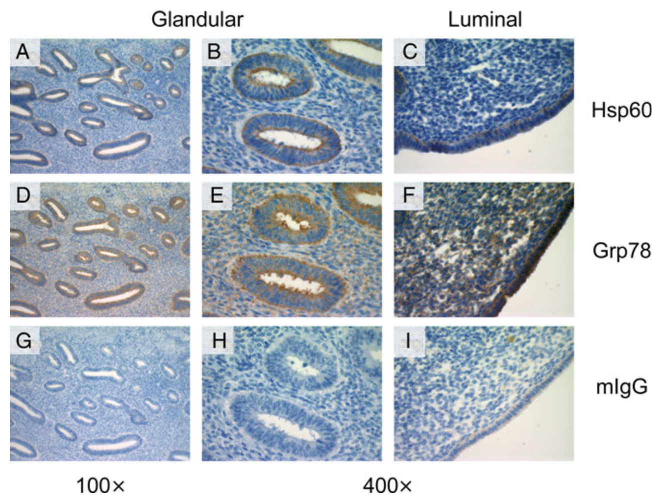


**Figure 1:** Immunohistochemical localization of Hsp60 (**A**–**C**) and Grp78 (**D**–**F**) proteins (brownish precipitate) in human endometrium Negative controls were obtained using commercial mouse IgG (mIgG; **G**–**I**) instead of monoclonal antibody

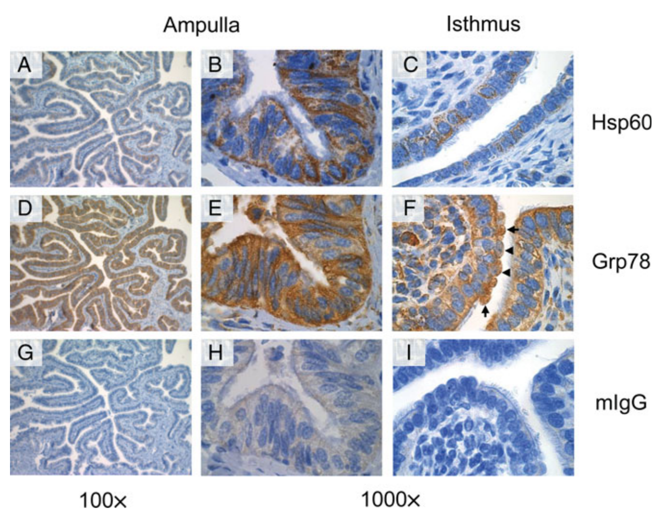


**Figure 2:** Immunohistochemical localization of Hsp60 (**A**–**C**) and Grp78 (**D**–**F**) proteins (brownish precipitate) in human Fallopian tube ampulla and isthmus sections

Negative controls were obtained using commercial mIgG (**G**–**I**) instead of monoclonal antibody. Arrows indicate emergent vesicles and arrowheads indicate shed nuclei positively stained for Grp78

### *Binding of recombinant Hsp60 and Grp78 to human spermatozoa*

The next step in our study was to determine whether recombinant Hsp60 or Grp78 bind to spermatozoa. Following sperm incubation with biotinylated Hsp60 and subsequent washes, a specific reactive band corresponding to biotinylated Hsp60 protein was detected in sperm protein extracts (Fig. 3A), suggesting that Hsp60 binds to the sperm surface. The specificity of this binding was strongly supported by the reduction in the band intensity when a 10-fold excess of non-biotinylated Hsp60 was added to the incubation medium. The specificity of the binding was also improved by removing BSA from the incubation medium (Fig. 3A). Using the same procedure, no conclusion could be made about sperm cell interaction with exogenous Grp78, as no specific band was observed using biotinylated Grp78 (Fig. 3A). As this could be due to a weak efficiency of the protein biotinylation procedure and therefore, a lower sensitivity of the assay, a His-tagged recombinant Grp78 protein was produced. Upon sperm incubation with this His-tagged protein, a reactive band corresponding to His-tagged Grp78 was detected using a monoclonal anti-His tag antibody (Fig. 3B). As with Hsp60, the specificity of this binding was supported by the reduction of the band intensity when a 10-fold excess of commercial Grp78 was added to the incubation medium (Fig. 3B), suggesting that Grp78 binds to the sperm surface.

### *Effect of Hsp60 and Grp78 on sperm acrosomal integrity and motility*

The influence of Hsp60 and Grp78 on human sperm capacitation was evaluated indirectly using the ratio of spontaneous and A23 187-induced acrosome reactions. After 4 h incubation, the percentage of spontaneous acrosome reactions remained unchanged in the presence of either Hsp60 or Grp78 ($5.7 \pm 0.8$ versus $5.2 \pm 0.9$ or $5.7 \pm 1.1$, respectively; $n = 9$). When the $Ca^{2+}$ ionophore A23 187 was added to sperm following the 4 h incubation, a significant increase in the percentages of acrosome reaction was observed, but this effect was not affected by the presence of Hsp60 or Grp78 in the incubation medium ($13.0 \pm 2.0$ versus $12.5 \pm 2.3$ or $12.5 \pm 1.5$, respectively; $n = 5$). In another set of experiments, the chaperones did not affect the spontaneous or ionophore-induced percentage of acrosome reactions in spermatozoa incubated for 4 h in the presence of the general phosphodiesterase inhibitor IBMX (data not shown).

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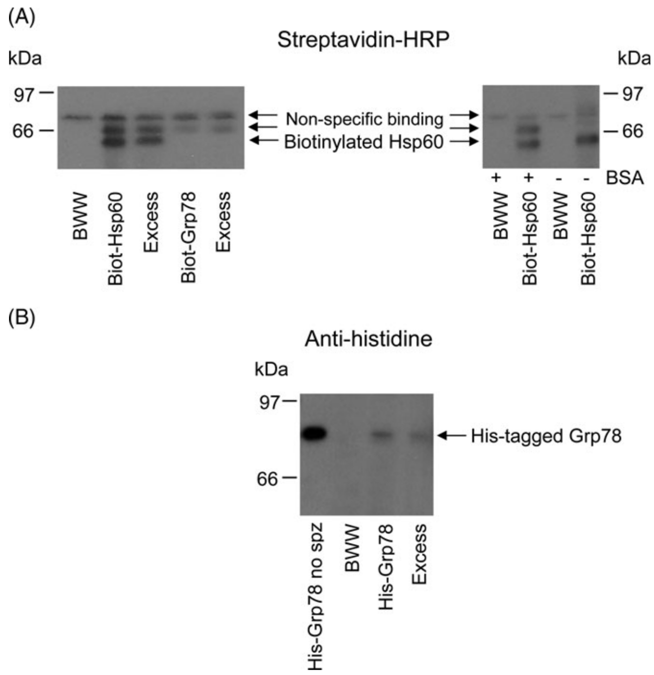


**Figure 3:** Binding of exogenous Hsp60 and Grp78 to human sperm (**A**) In the left panel, percoll-washed spermatozoa were incubated for 1 h at 37°C in BSA-containing BWW in the absence (lane 1) or presence of 100 ng/ml of biotinylated Hsp60 (lanes 2 and 3) or Grp78 (lane 4 and 5). A 10-fold excess of non-biotinylated Hsp60 or Grp78 was added to one incubation tube (lanes 3 and 5, respectively). In the right panel, sperm cells were incubated under the same conditions as above in BWW supplemented or not with BSA (lanes 1 and 2 and lanes 3 and 4, respectively) in the absence (lanes 1 and 3) or presence of 100 ng/ml of biotinylated Hsp60 (lanes 2 and 4). Proteins extracted from $1 \times 10^6$ sperm cells were processed by SDS-PAGE and transferred to nitrocellulose membranes. The presence of biotinylated proteins was revealed by streptavidin-HRP. Of the three bands detected for biotinylated Hsp60, streptavidin bound non-specifically to the highest band (detected in the absence of biotinylated proteins) and to a reactive band at 66 kDa which was present only when BSA was included in the incubation medium. (**B**) Sperm cells were incubated in BSA-free BWW in the absence (lane 2) or presence (lanes 3 and 4) of 100 ng/ml of histidine-tagged recombinant Grp78. A 10-fold excess of non-tagged Grp78 was added to one tube (lane 4). His-tagged recombinant Grp78 protein (20 ng) is shown as a positive control (lane 1). The reactive band at 83 kDa observed in lane 3 and 4 correspond to the molecular weight of the His-tagged recombinant protein. Molecular weight markers (kDa) are indicated. ($n = 4$)

As with acrosomal integrity, neither Hsp60 nor Grp78 affected the percentages of motile sperm ($55.1 \pm 5.3$ versus $58.1 \pm 5.0$ or $58.0 \pm 5.9$, for control, Hsp60 and Grp78, respectively; $n = 4$). Similarly, no effect of exogenous molecular chaperones was observed on the percentage of progressive motility, curvilinear velocity and amplitude of lateral head displacement. When the phosphodiesterase inhibitor IBMX was added to the incubation medium, a significant increase ($P < 0.05$) in the percentage of motility was observed following the 4 h incubation ($68.9 \pm 3.3$ versus $55.1 \pm 5.3$; $n = 4$). However, this increase was not significant when either Hsp60 or Grp78 was present in the incubation medium ($63.1 \pm 4.3$ and $63.0 \pm 7.1$ compared with $58.1 \pm 5.0$ and $58.0 \pm 5.9$, respectively). None of the other motility parameters such as beat cross frequency, the percentage of straightness and the percentage of linearity were modified by the presence of IBMX, Hsp60 or Grp78 (data not shown).

### *Effect of Hsp60 and Grp78 on sperm protein tyrosine phosphorylation*

As reported in previous studies, an increase in the phosphotyrosine content of p81 and p105, the two major human sperm phosphotyrosine-containing proteins, was observed following incubation under capacitating conditions (Fig. 4A). These two proteins were used as markers in the present study to determine the effect of Hsp60 and Grp78 on sperm protein tyrosine phosphorylation. As commonly reported in the literature, inclusion of the phosphodiesterase inhibitor IBMX in the incubation medium provoked a significant increase in the phosphotyrosine content of p81 and p105 (Fig. 4A). Therefore, the effects of recombinant Hsp60 and Grp78 on the phosphotyrosine content of the p81 and p105 were investigated on sperm incubated in the absence or presence of IBMX.

Different effects of recombinant Hsp60 and Grp78 on protein tyrosine phosphorylation were observed whether or not IBMX was present in the incubation medium. No significant modification of the p81 and p105 phosphotyrosine content was observed when sperm were incubated in BWW medium devoid of IBMX but supplemented with either

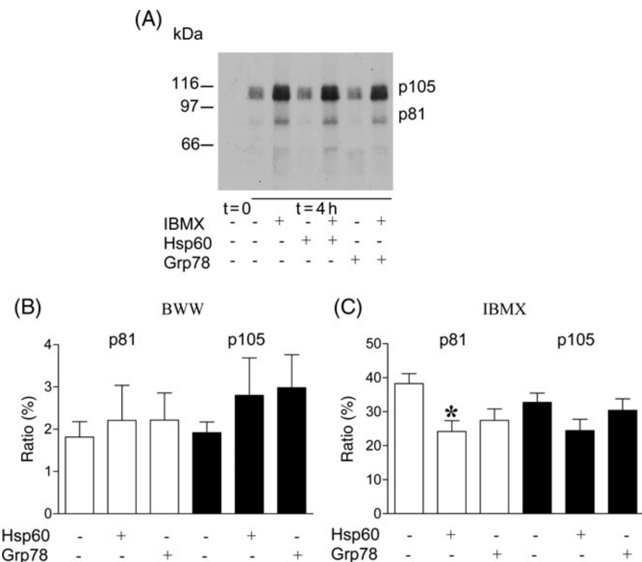


**Figure 4:** Effect of Hsp60 and Grp78 on tyrosine phosphorylation of sperm proteins

Percoll-washed spermatozoa were incubated 4 h at 37°C with 10 ng/ml of either Hsp60 or Grp78 in BWW containing 500 μM IBMX or DMSO as the solvent control for IBMX. Spermatozoa were washed; proteins were solubilized, separated by SDS-PAGE and electrotransfered on nitrocellulose membrane as described in ‘Materials and Methods’. The membrane was probed first with anti-phosphotyrosine antibody and then with anti-tubuline antibody to ensure that equal amounts of sperm proteins were applied on the gel. (**A**) representative anti-phosphotyrosine blot; (**B**) normalized signal of the phosphotyrosine content of p81 (open bars) and of p105 (close bars) to the α-tubulin content measured in each treatment by densitometric analysis; (**C**) normalized signal for the phosphotyrosine content in presence of IBMX in incubation media as described in B). *$P < 0.05$ versus control incubation with IBMX ($n = 3$)



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Hsp60 or Grp78 (Fig. 4B). However, the phosphotyrosine content of p81 and p105 increased when the incubation medium contained IBMX (Fig. 4A). The level of p81 phosphotyrosine content was significantly lower when Hsp60 was present in the IBMX-supplemented medium ($P < 0.05$, Fig. 4C). The p81 phosphotyrosine content was also lower when Grp78 was present in the IBMX-containing medium, but the difference was not significant. Neither Hsp60 nor Grp78 significantly affected the increase in p105 tyrosine phosphorylation induced by IBMX.

### *Effect of hsp60 and grp78 on intracellular calcium*

As expected, an increase in free cytosolic $Ca^{2+}$ concentration was measured when sperm were incubated for 4 h in the BWW medium ($P < 0.001$, Fig. 5). When recombinant Hsp60 or Grp78 proteins (10 ng/ml) were added to the incubation medium, a further increase in the VRE ($Ca^{2+}$-bound/unbound INDO-1 probe) was measured (8.4 and 9.9%, for Hsp60 and Grp78, respectively; $P < 0.05$).

At the end of the incubation, sperm were challenged with thapsigargin as an indicator of the amount of $Ca^{2+}$ loaded in intracellular stores. Thapsigargin induced a $Ca^{2+}$ increase that plateaued within 1 min (Fig. 6A). When sperm were incubated with Grp78, the increase occurred in the same time-dependent manner but the intracellular $Ca^{2+}$ levels measured at the plateau were 9.3% higher ($P = 0.05$), while Hsp60 had no effect (Fig. 6B). The effect of progesterone on sperm intracellular $Ca^{2+}$ concentration was similar to, what is generally observed and reported in the literature (Fig. 6C): a rapid (~30 s) and significant increase in intracellular $Ca^{2+}$ followed by a constant decrease to reach a plateau above the basal level (Blackmore *et al.*, 1990). Violet/blue ratios measured at the peak were used to compare sperm responsiveness to progesterone. Both Hsp60 and Grp78 further elevated the intracellular $Ca^{2+}$ levels reached by sperm upon progesterone treatment. The cytosolic $Ca^{2+}$ levels at the peak were 6.9% higher when sperm were incubated with Hsp60 and 8.9% higher when sperm were incubated with Grp78 when compared with the ratios obtained with sperm incubated under control conditions ($P < 0.05$ and $P < 0.01$, respectively, Fig. 6D). As

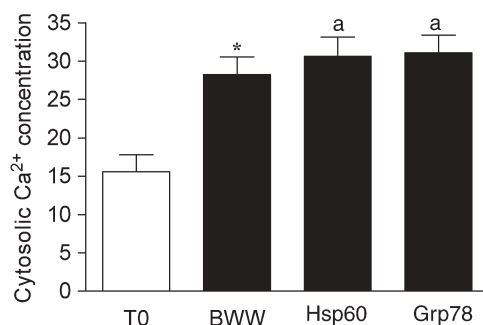


**Figure 5:** Effect of Hsp60 and Grp78 on intracellular cytosolic calcium levels. INDO-1 AM loaded spermatozoa were incubated for 4 h at 37°C in the absence or presence of either Hsp60 or Grp78 or solvent control (BWW), or left unincubated (T0). The evaluation of VRE was performed by flow cytometry as described in the 'Materials and Methods'. The basal cytosolic $Ca^{2+}$ concentration was measured for 1 min. $^{*}P < 0.001$ versus T0 and $^{a}P < 0.05$ versus BWW ($n = 6$)

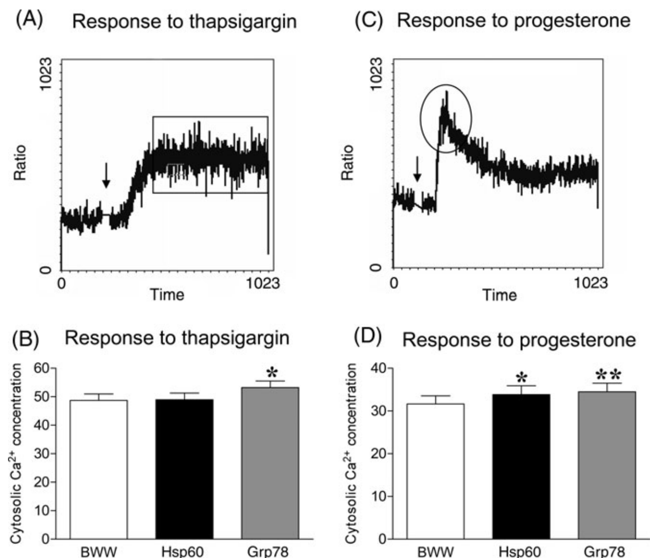


**Figure 6:** Effect of Hsp60 and Grp78 on thapsigargin- and progesterone-induced increase in sperm intracellular $Ca^{2+}$ concentration
INDO-1 AM loaded spermatozoa were incubated for 4 h at 37°C in the absence or presence of either Hsp60 or Grp78 or solvent control (BWW). The evaluation of VRE was performed by flow cytometry as described in the 'Materials and Methods'. After a 1 min measurement of the basal cytosolic $Ca^{2+}$ concentration, (**A**) 5 μM thapsigargin was added and the measurement was performed for a further 4 min. The VRE measured at the plateau (boxed region) following thapsigargin addition is shown in (**B**). After 1 min measurement of the basal cytosolic $Ca^{2+}$ concentration, (**C**) 10 μm progesterone was added and the measurement was performed for a further 4 min. The VRE measured at the peak (circle region) following progesterone addition is presented in (**D**). (A and C) are representative plots of VRE from six independent experiences with six different sperm donors. $^{*}P < 0.05$ versus BWW and $^{**}P < 0.01$ versus BWW ($n = 6$)

stated in the 'Materials and Methods' section, measurements of intracellular $Ca^{2+}$ concentration were done strictly on sperm that did not incorporate PI.

Under the conditions used in this study, sperm viability, evaluated by the PI incorporation during the intracellular $Ca^{2+}$ assessment, was maintained during the 4 h incubation ranging from 94% at $t = 0$ to 90–92% at $t = 4$ h (data not shown). Neither Hsp60 nor Grp78 had a significant effect on sperm viability (data not shown).

## Discussion

In the present study, we evaluated the effect of two recombinant chaperone proteins, Hsp60 and Grp78, on human sperm functions. These chaperone proteins did not significantly affect some of the physiological changes of human sperm cells related to capacitation such as motility and acrosome integrity. On the other hand, recombinant Hsp60 and Grp78 modulated other notable sperm parameters, including the appearance of a capacitation-associated phosphotyrosine containing protein stimulated by IBMX and the intracellular cytosolic calcium levels. Therefore, the present study clearly demonstrates that exogenous chaperone proteins can affect human sperm physiology in the process of the acquisition of fertilizing ability.

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*In vivo*, spermatozoa must interact with cervical mucus, endometrial and oviductal epithelial cells before ultimately reaching the oocyte (De Jonge, 2005). In order to mimic natural conditions *in vitro*, sperm are incubated in the presence of either whole endometrial or oviductal cells, or their secretions (Barratt and Cooke, 1991; Quintero *et al.*, 2005). Nonetheless, few studies have investigated the effect of specific molecules secreted or present on the surface of epithelial cells from the female genital tract on sperm functions. In the present study, it is shown that both Hsp60 and Grp78 are present in the columnar epithelia of uterus and Fallopian tube sections and are detected in the apical area of these cells. This is in perfect agreement with the surface expression of Hsp60 and Grp78 in bovine and swine OEC plasma membrane (Boilard *et al.*, 2004; Sostaric *et al.*, 2006) and supports potential interactions between these factors and human spermatozoa during their transit within the female reproductive tract. In fact, our data show that recombinant Hsp60 and Grp78 associate with human spermatozoa, which is in agreement with the exchange of Hsp60 and Grp78 observed between bovine OEC apical membranes and bull sperm (Boilard *et al.*, 2004).

There are different hypotheses on the role of oviducts on spermatozoa. On one hand, oviduct cells and secretions confer to sperm or facilitate their acquisition of the ability to interact with and fertilize the oocyte. On the other hand, cells and secretions from the female genital tract reduce sperm metabolism, prolong their viability and retard/prevent their ability to interact with the oocyte. In fact, these two opposite roles are present in the female reproductive tract but may take place in different areas. One of the objectives of this study was to determine the role of the two chaperone proteins, Hsp60 and Grp78, according to the duality of the oviduct action on sperm.

Sperm motility patterns change during capacitation with an increase in hyperactivity. This new motility pattern is characterized by an increase in sperm velocity and lateral head displacement, in association with a decrease in displacement linearity. In the present study, none of the two chaperones investigated affected any of the motility parameters. Phosphodiesterase inhibitors have been shown for many years to promote or accelerate the onset of sperm capacitation and to affect sperm motility. In this study, Hsp60 and Grp78 partially antagonized the stimulatory effect of IBMX on the percentage of sperm motility. Otherwise, no other sperm motility parameters were influenced by Hsp60 or Grp78.

An increases in protein tyrosine phosphorylation is known to be a key intracellular event associated with sperm capacitation (Leclerc *et al.*, 1996,1997,1998; de Lamirande *et al.*, 1997; Visconti *et al.*, 2002). In the present study, p81 and p105 were considered as good markers to evaluate the effect of Hsp60 and Grp78 on human sperm protein tyrosine phosphorylation. The phosphotyrosine contents of p81 and p105 were not affected when spermatozoa were incubated with Hsp60 or Grp78 alone. When protein tyrosine phosphorylation was activated by the phosphodiesterase inhibitor IBMX, Hsp60 partially, although significantly, prevented this effect. The blocking effect of Grp78 was not as stringent as Hsp60. These findings suggest that Hsp60 and Grp78 may counteract the cAMP signalling pathway although the specific target remains to be identified. Inhibitors of ser/thr phosphatases such Okadaic acid or calyculin A have been shown to enhance the phosphotyrosine content of sperm p81 and p105 (Leclerc *et al.*, 1996). Whether Hsp60 or Grp78, this latter being known as an activator of the protein phosphatase PP1 (Chun *et al.*, 1999), prevent IBMX-stimulated sperm tyrosine phosphorylation through this mechanism remains to be further investigated.

$Ca^{2+}$ influx is a key biochemical event that occurs during capacitation and $Ca^{2+}$ is required for capacitation and induction of the acrosome reaction (Florman *et al.*, 1992; DasGupta *et al.*, 1993). In the present study, free cytosolic $Ca^{2+}$ reached higher levels when sperm were incubated in the presence of recombinant chaperones. Thapsigargin causes an increase in cytosolic $Ca^{2+}$ concentration through mobilization from intracellular stores, which in turn promotes a $Ca^{2+}$ influx from the extracellular medium (Meizel and Turner, 1993). Therefore, the higher $Ca^{2+}$ levels measured when sperm were incubated with Grp78 prior to the thapsigargin treatment may indicate that more $Ca^{2+}$ was accumulated within intracellular stores or that the secondary influx, occurring after store depletion, was facilitated. Similarly, higher $Ca^{2+}$ levels were measured in response to progesterone when sperm were incubated with either exogenous Grp78 or Hsp60. These results confirm those obtained with thapsigargin. Our data, however, do not allow discrimination between an involvement of these two chaperone proteins in the intracellular storage of $Ca^{2+}$, its mobilization or the activation of a secondary $Ca^{2+}$ influx from the external medium.

Extracellular functions of chaperone proteins have been under intense investigation as a result of the increasing accessibility of recombinant protein (Tsan and Gao, 2004). Exogenous HSPs induce protein expression by various transcription and translation processes in different cell types. Recent studies reveal that HSPs are potent activators of the innate immune system by induction of cytokine production (Tsan and Gao, 2004). Molecular chaperones activate human monocytes (Bethke *et al.*, 2002) and stimulate IL-6 production in macrophages (Gobert *et al.*, 2004) and treatment of T cells with Hsp60 can regulate inflammation, inhibit chemotaxis and up-regulate suppressor of cytokine signalling (SOCS)3 via toll like receptor (TLR)2 and signal transducer and activator of transcription (STAT)3 activation (Zanin-Zhorov *et al.*, 2003). Unlike somatic cells, spermatozoa are transcriptionally inactive and so many of the reported pathways are not likely to occur as a response to the presence of exogenous chaperones. Studies are currently in progress to elucidate how the $Ca^{2+}$ and protein phosphotyrosine content of human sperm are modulated by the recombinant molecular chaperones Hsp60 and Grp78 during capacitation.

Commercial recombinant Hsp60 or Grp78 did not drastically affect sperm functions. Although these results seem inconsistent, there is no consensus regarding the effect of OEC or their secretions on sperm capacitation occurring during the transit of spermatozoa in the female reproductive tract. Most studies have shown that OEC components maintain motility but their effects on sperm membrane integrity and viability parameters

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are variable. OEC are reported to maintain sperm motility, prevent human sperm capacitation (Murray and Smith, 1997) and maintain low intracellular calcium concentration (Smith, 1998), while other studies report a promoting effect of OEC on sperm capacitation and hyperactivation (Kervancioglu *et al.*, 1994,2000). In the present study, the parameters were evaluated after a 4 h incubation. Therefore, it is not known whether a stronger effect would have occurred earlier during the incubation, which would indicate that the chaperones accelerated the onset of sperm capacitation. On the other hand, a longer incubation period may have allowed the assessment of a stronger effect of the chaperone proteins on sperm viability, motility and membrane/acrosome integrity.

The present study reports the potential of two individual chaperone proteins, Hsp60 and Grp78, both expressed by OEC to modulate motility, protein tyrosine phosphorylation and intracellular calcium levels of sperm during capacitation. Further investigation is required to determine the signalling pathways triggered by these proteins and to identify potent chaperone-interacting proteins on the sperm surface.

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## References

Baillie HS, Pacey AA, Warren MA, Scudamore IW, Barratt CL. Greater numbers of human spermatozoa associate with endosalpingeal cells derived from the isthmus compared with those from the ampulla. *Hum Reprod* 1997;**12**:1985–1992.

Barratt CL, Cooke ID. Sperm transport in the human female reproductive tract–a dynamic interaction. *Int J Androl* 1991;**14**:394–411.

Bethke K, Staib F, Distler M, Schmitt U, Jonuleit H, Enk AH, Galle PR, Heike M. Different efficiency of heat shock proteins (HSP) to activate human monocytes and dendritic cells: superiority of HSP60. *J Immunol* 2002;**169**:6141–6148.

Biggers JD, Whitten WK, Whittingham DG. The culture of mouse embryos in vitro. In: Daniel JC Jr (ed.). *Methods of Mammalian Embryology*. San Fransisco: WH Freeman, 1971, 86–116.

Blackmore PF, Beebe SJ, Danforth DR, Alexander N. Progesterone and 17 alpha-hydroxyprogesterone. Novel stimulators of calcium influx in human sperm. *J Biol Chem* 1990;**265**:1376–1380.

Boilard M, Bailey J, Collin S, Dufour M, Sirard MA. Effect of bovine oviduct epithelial cell apical plasma membranes on sperm function assessed by a novel flow cytometric approach. *Biol Reprod* 2002;**67**:1125–1132.

Boilard M, Reyes-Moreno C, Lachance C, Massicotte L, Bailey JL, Sirard MA, Leclerc P. Localization of the chaperone proteins GRP78 and HSP60 on the luminal surface of bovine oviduct epithelial cells and their association with spermatozoa. *Biol Reprod* 2004;**71**:1879–1889.

Chun YS, Park JW, Kim MS, Shima H, Nagao M, Lee SH, Park SW, Chung MH. Role of the 78-kDa glucose-regulated protein as an activity modulator of protein phosphatase1gamma2. *Biochem Biophys Res Commun* 1999;**259**:300–304.

Cross NL, Morales P, Overstreet JW, Hanson FW. Two simple methods for detecting acrosome-reacted human sperm. *Gamete Res* 1986;**15**:213–226.

DasGupta S, Mills CL, Fraser LR. Ca(2+)-related changes in the capacitation state of human spermatozoa assessed by a chlortetracycline fluorescence assay. *J Reprod Fertil* 1993;**99**:135–143.

De Jonge C. Biological basis for human capacitation. *Hum Reprod Update* 2005;**11**:205–214.

de Lamirande E, Leclerc P, Gagnon C. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Mol Hum Reprod* 1997;**3**:175–194.

Dorval V, Dufour M, Leclerc P. Regulation of the phosphotyrosine content of human sperm proteins by intracellular Ca2+: role of Ca2+-adenosine triphosphatases. *Biol Reprod* 2002;**67**:1538–1545.

Ellington JE, Ignotz GG, Varner DD, Marcucio RS, Mathison P, Ball BA. In vitro interaction between oviduct epithelial and equine sperm. *Arch Androl* 1993;**31**:79–86.

Ellington JE, Jones AE, Davitt CM, Schneider CS, Brisbois RS, Hiss GA, Wright RWJ. Human sperm function in co-culture with human, macaque or bovine oviduct epithelial cell monolayers. *Hum Reprod* 1998;**13**:2797–2804.

Ellington JE, Evenson DP, Wright RWJ, Jones AE, Schneider CS, Hiss GA, Brisbois RS. Higher-quality human sperm in a sample selectively attach to oviduct (fallopian tube) epithelial cells in vitro. *Fertil Steril* 1999a;**71**:924–929.

Ellington JE, Samper JC, Jones AE, Oliver SA, Burnett KM, Wright RW. In vitro interactions of cryopreserved stallion spermatozoa and oviduct (uterine tube) epithelial cells or their secretory products. *Anim Reprod Sci* 1999b;**56**:51–65.

Florman HM, Corron ME, Kim TD, Babcock DF. Activation of voltage-dependent calcium channels of mammalian sperm is required for zona pellucida-induced acrosomal exocytosis. *Dev Biol* 1992;**152**:304–314.

Gobert AP, Bambou JC, Werts C, Balloy V, Chignard M, Moran AP, Ferrero RL. Helicobacter pylori heat shock protein 60 mediates interleukin-6 production by macrophages via a toll-like receptor (TLR)-2-, TLR-4-, and myeloid differentiation factor 88-independent mechanism. *J Biol Chem* 2004;**279**:245–250.

Gualtieri R, Boni R, Tosti E, Zagami M, Talevi R. Intracellular calcium and protein tyrosine phosphorylation during the release of bovine sperm adhering to the fallopian tube epithelium in vitro. *Reproduction* 2005;**129**:51–60.

Kervancioglu ME, Djahanbakhch O, Aitken RJ. Epithelial cell coculture and the induction of sperm capacitation. *Fertil Steril* 1994;**61**:1103–1108.

Kervancioglu ME, Saridogan E, Aitken RJ, Djahanbakhch O. Importance of sperm-to-epithelial cell contact for the capacitation of human spermatozoa in fallopian tube epithelial cell cocultures. *Fertil Steril* 2000;**74**:780–784.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;**227**:680–685.

Laflamme J, Akoum A, Leclerc P. Induction of human sperm capacitation and protein tyrosine phosphorylation by endometrial cells and interleukin-6. *Mol Hum Reprod* 2005;**11**:141–150.

Leclerc P, de Lamirande E, Gagnon C. Cyclic adenosine 3′,5′monophosphate-dependent regulation of protein tyrosine phosphorylation in relation to human sperm capacitation and motility. *Biol Reprod* 1996;**55**:684–692.

Leclerc P, de Lamirande E, Gagnon C. Regulation of protein-tyrosine phosphorylation and human sperm capacitation by reactive oxygen derivatives. *Free Radic Biol Med* 1997;**22**:643–656.

Leclerc P, de Lamirande E, Gagnon C. Interaction between Ca2+, cyclic 3′,5′ adenosine monophosphate, the superoxide anion, and tyrosine phosphorylation pathways in the regulation of human sperm capacitation. *J Androl* 1998;**19**:434–443.

Meizel S, Turner KO. Initiation of the human sperm acrosome reaction by thapsigargin. *J Exp Zool* 1993;**267**:350–355.

Murray SC, Smith TT. Sperm interaction with fallopian tube apical membrane enhances sperm motility and delays capacitation. *Fertil Steril* 1997;**68**:351–357.

Quintero I, Ghersevich S, Caille A, Munuce MJ, Daniele SM, Morisoli L. Effects of human oviductal in vitro secretion on spermatozoa and search of sperm-oviductal proteins interactions. *Int J Androl* 2005;**28**:137–143.

Smith TT. The modulation of sperm function by the oviductal epithelium. *Biol Reprod* 1998;**58**:1102–1104.

Sostaric E, Georgiou AS, Wong CH, Watson PF, Holt WV, Fazeli A. Global profiling of surface plasma membrane proteome of oviductal epithelial cells. *J Proteome Res* 2006;**5**:3029–3037.

Suarez SS. Formation of a reservoir of sperm in the oviduct. *Reprod Domest Anim* 2002;**37**:140–143.

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Tabibzadeh S, Kong QF, Satyaswaroop PG, Babaknia A. Heat shock proteins in human endometrium throughout the menstrual cycle. *Hum Reprod* 1996;**11**:633–640.

Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979;**76**: 4350–4354.

Tsan MF, Gao B. Cytokine function of heat shock proteins. *Am J Physiol Cell Physiol* 2004;**286**:C739–C744.

Visconti PE, Westbrook VA, Chertihin O, Demarco I, Sleight S, Diekman AB. Novel signaling pathways involved in sperm acquisition of fertilizing capacity. *J Reprod Immunol* 2002;**53**:133–150.

Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD (eds). *The Physiologie of Reproduction.* New York: Raven Press, 1994, 189–317.

Yeung WS, Ng VK, Lau EY, Ho PC. Human oviductal cells and their conditioned medium maintain the motility and hyperactivation of human spermatozoa in vitro. *Hum Reprod* 1994;**9**:656–660.

Zanin-Zhorov A, Nussbaum G, Franitza S, Cohen IR, Lider O. T cells respond to heat shock protein 60 via TLR2: activation of adhesion and inhibition of chemokine receptors. *Faseb J* 2003;**17**:1567–1569.

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