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Sperm Sorting Procedure Induces a Redistribution of Hsp70 but Not Hsp60 and Hsp90 in Boar Spermatozoa

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Abstract

Heat shock proteins, besides their protective function against stresses, have been recently indicated as key factors for sperm fertilizing ability. Since sexing sperm by high-speed flow-cytometry subjects them to different physical, mechanical, and chemical stresses, the present study was designed to verify, by immunofluorescence and Western blot, whether the sorting procedure induces any modification in the amount and cellular distribution of heat shock proteins 60, 70, and 90 (Hsp60, Hsp70, Hsp90). Immunolocalization and Western blot quantification of both Hsp60 and Hsp90 did not reveal differences between unsorted and sorted semen. On the contrary, a redistribution of Hsp70 immunoreactivity from the equatorial subsegment toward the equator of sperm cells was recorded after sorting; this relocation suggests capacitation-like changes of sperm membrane. This modification seems to be caused mainly by incubation with Hoechst 33342, while both passage of sperm through flow cytometer and laser beam represent only minor stimuli. A further Hsp70 redistribution seems to be due to the final steps of sperm sorting, charging, and deflection of drops, and to the dilution during collection. On the other hand, staining procedure and mechanical stress seem to be the factors most injurious to sperm viability. Moreover, Hsp70 relocation was deeply influenced by the storage method. In fact, storing sexed spermatozoa, after centrifugation, in a small volume in presence of seminal plasma induced a reversion of Hsp70 redistribution, while storage in the diluted

catch fluid of collection tubes caused Hsp70 relocation in most sorted spermatozoa.

Key words: Flow cytometry, heat shock protein 60, heat shock protein 70, heat shock protein 90, pig

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Xand Y chromosome-bearing mammalian spermatozoa can be separated with higher than 90% accuracy by using a flow-cytometric sperm sorter on the basis of DNA content. The method is based on staining sperm with a DNA-binding fluorochrome, Hoechst 33342, and flow-cytometrically sorting them in 2 populations enriched for X- or Y-bearing cells (reviewed in Johnson et al, 2005 and Garner, 2006).

Sexing sperm by high-speed flow cytometry subjects them to different stresses, such as high dilution, Hoechst nuclear staining, high pressure, mechanical forces associated with passage through the sorter, exposure to UV laser beam, electrical charge, and projection into the collection tube at high speed (Maxwell et al, 1996; Maxwell and Johnson, 1997; Maxwell and Johnson, 1999; Johnson, 2000; Garner and Shu, 2002; Vazquez et al, 2002; Parrilla et al, 2005; Shu et al, 2005; Spinaci et al, 2005a; De Ambrogi et al, 2006).

Mammalian cells respond to environmental stresses (such as exposure to high temperature, toxic chemicals, or various physical conditions) by the synthesis of a conserved family of proteins known as heat shock proteins (HSPs), even if these proteins have also been reported to be present in nonshocked tissues (Welch, 1992; Morimoto et al, 1994). These molecular chaperone proteins have been shown to be involved in maintaining proper protein conformation, stabilizing unfolded precursor proteins prior to their assembly into macromolecular complexes, and participating in protein trafficking and translocation through membranes (Gething and Sambrook, 1992).

The highly differentiated sperm cell seems to be transcriptionally inactive and to lack the translational machinery within the residual cytoplasm. Sperm HSPs are probably synthesized only during spermatogenesis, prior to chromatin condensation. For this reason sperm cells cannot respond to stress with an enhanced HSP expression but could only consume the already synthesized HSP pool.

HSPs, besides their protective function against stresses, have been recently indicated as important key factors for sperm fertilizing ability. In human medicine, a potential role of the immune response to Hsp60 and Hsp70 in cases of male infertility has been suggested (Eggert-Kruse et al, 2002; Bohring and Krause, 2003). A positive correlation between Hsp70 levels and quality traits has been described in boar by Huang et al (2000a); moreover, a role of this protein during pig and bovine gamete interaction has been recently demonstrated (Matwee et al, 2001; Spinaci et al, 2005b). Hsp60, a protein that mediates correct folding of mitochondrial proteins, has been recently immunolocalized in pig spermatozoa (Volpe et al, 2006). A crucial role of Hsp90 in boar sperm motility regulation has been hypothesized: a decrease in sperm Hsp90 levels precedes, in fact, the decline of sperm motility during cooling, and geldanamycin, an Hsp90-specific inhibitor, has been demonstrated to impair porcine sperm motility (Huang et al, 1999; Huang et al, 2000b).

The aim of this study was to verify whether the sorting process induces any modification in Hsp60, Hsp70, and Hsp90 distribution and/or content in boar spermatozoa. Moreover, the impact of sorting steps on sperm membrane has been evaluated on the basis of Hsp70 redistribution.

Materials and Methods

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Animals were housed and handled according to EEC animal care guidelines.

All the reagents were obtained from Sigma Chemical Co (St Louis, Mo) unless otherwise specified.

Semen Collection and Preparation

Sperm-rich fractions of ejaculates were collected by glovedhand technique from 3 mature boars of proven fertility and extended in equal volumes of Androhep (Minitüb, Tiefenbach, Germany). In order to minimize the boar effect, samples were pooled. Samples were diluted with Androhep to 100 x 10^6 spermatozoa/mL. Aliquots of 1 mL of diluted semen were then transferred into Falcon tubes and stained with 10 µL of 5 mg/mL Hoechst 33342 stock solution for 1 hour at 35 °C in the dark. This stain concentration was chosen because it usually ensures the best separation pattern for sorting under our laboratory conditions.

Just prior to sorting, 1 µL of food dye (FD&C ≥40, Warner Jenkinson, St Louis, Mo) stock solution (25 mg/mL) was added to each sample to quench the Hoechst 33342 fluorescence of spermatozoa with damaged membrane in order to exclude them from the sorting process by dead-cell gating. The samples were then filtered through a 60-µm nylon mesh filter to remove debris or clumped spermatozoa.

Flow Sorting

A MoFlo SX flow cytometer/sperm sorter (DakoCytomation Inc, Fort Collins, Colo) equipped with an argon laser (wavelength 351 at 150 mW) and modified especially for sorting sperm (Johnson and Pinkel, 1986; Johnson and Welch, 1999) was used. Dulbecco phosphate buffered saline (DPBS) served as sheath fluid. The instrument sheath pressure was 40 psi, and the trigger rate was adjusted to 20 000 cells per second. Sorted spermatozoa were deflected into 20-mL polypropylene tubes containing 500 µL of 2% Tes-Tris-egg yolk buffer (Johnson, 1991) supplemented with 10 µL of frozen-thawed boar seminal plasma. After collection of 7–8 x 10^6 sperm per tube, the 2 populations were pooled (since sex predetermination was not an objective of the experiment). The samples were centrifuged at 800 x *g* for 10 minutes, and the pellet was resuspended with Androhep. Spermatozoa were counted and 1 aliquot containing 5 x 10^6 cells was centrifuged at 800 x *g* for 10 minutes, the supernatant was discarded, and the final pellet was frozen until Western blot assay. The remaining sperm cells were fixed as below described for immunofluorescence staining. Control semen was kept at 16 °C until processed.

Immunofluorescence Staining

All the procedures were carried out at room temperature unless otherwise specified.

Sperm cells were spotted onto poly-L-lysine-coated slides and fixed with methanol at −20 °C for 5 minutes and then with acetone for 30 seconds. The slides were then washed with PBS and blocked with 10% (v/v) fetal calf serum in PBS for at least 30 minutes. Antibody dilutions were performed in blocking solution. Monoclonal anti-Hsp60 antibody (Lk-1 mAb; StressGen Biotechnologies Corp, Victoria, Canada), anti-Hsp70 antibody (C92F3A-5 mAb; StressGen) or anti-Hsp90 antibody (AC88 mAb; StressGen) were added at 1:200 dilution. Incubation was carried out overnight (15–18 hours) at 4 °C. After extensive washing with PBS, sperm cells were incubated with a sheep-anti-mouse FITC-conjugated secondary antibody (1:800) for 1 hour in the dark. Slides were washed with PBS and mounted with Vectashield mounting medium containing propidium iodide counterstain for DNA (Vector Laboratories, Burlingame, Calif). Control cells were treated similarly, with the omission of primary antiserum. Spermatozoa were evaluated with a Nikon Eclipse E600 microscope equipped with an Ex 465–495 DM 505 BA 515–555 filter for the detection of green fluorescence (FITC) and an EX 540 DM 565 BA 605–655 filter for red fluorescence (PI) and a Nikon DXM 1200 digital camera provided with ACT-2U software for DMX 1200.

Western Blot

For protein analysis, spermatozoa were washed with PBS and centrifuged at 800 x *g* for 3 minutes. The pellet was then resuspended in SDS buffer (Tris-HCl 62.5 mmol pH 6.8; SDS 2%, glycerol 20%). Proteins from 1 x 10^6 spermatozoa were separated by NuPage 10% Bis-Tris Gel (Gibco-Invitrogen, Paisley, United Kingdom) for 50 minutes at 200 V, then were electrophoretically transferred onto a nitrocellulose membrane. Blots were washed in PBS, and protein transfer was checked by staining both the nitrocellulose membranes with 0.2% Ponceau Red and the gels with Coomassie Blue. Nonspecific protein binding on nitrocellulose membranes was blocked with 5% milk powder in PBS-T20 (PBS 0.1% Tween 20) for 1 hour at room temperature. The membranes were then incubated with a 1:1000 dilution of the anti-Hsp60, anti-Hsp70, or anti-Hsp90 monoclonal antibodies (StressGen) in Tris buffered saline T20 (20 mmol Tris-HCl, pH 7.4, 500 mmol NaCl, 0.1% T20) overnight at 4 °C. After several washings with PBS-T20, the membranes were incubated at first with a 1:10 000 dilution of a goat conjugate anti-mouse IgG antiserum (StressGen) and then with a 1:1000 dilution of a horseradish peroxidase (HRP)-linked antibiotin antibody. The Western blots were developed using chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate; Pierce, Rockford, Ill) according to the manufacturer's instructions. The intensity of luminescent signal of the resultant bands was recorded by Fluor-S Multimager using the Quantity One software package (Bio-Rad Laboratories Inc, Hercules, Calif). In order to normalize the Hsp data on housekeeping protein, membranes were stripped (briefly: membranes were washed 5 minutes in water, then 5 minutes in 0.2 mol NaOH, and washed again in water) and reprobed for the housekeeping ß-tubulin (1:500 sc-5274; Santa Cruz Biotechnology Inc, Santa Cruz, Calif). The relative protein content (Hsp/ß-tubulin) was expressed in arbitrary units (AU).

Effect of the Different Steps of the Sorting Procedure

Hsp70 Relocalization— In order to evaluate the sorting step(s) responsible for Hsp70 redistribution, Hsp70 immunolabeling was performed as above described on sperm cells after 1) Hoechst 33342 staining (Stained group), 2) staining and passage through the sperm sorter with the laser switched off (No Laser group), 3) staining and passing through the sperm sorter with the laser on (Laser group) (the sperm cells of both No Laser and Laser groups were collected in 500 µL of 2% Tes-Tris-egg yolk buffer supplemented with 10 µL of frozen-thawed seminal plasma directly from the central stream before they fell into the waste, therefore including also cells normally discarded from sex sorting by dead gating), and 4) the whole sorting procedure, deflecting all (live and dead) sperm cells (All Sort group). After collection, all the samples were centrifuged at 800 x *g* for 10 minutes, resuspended with Androhep, and then fixed for immunofluorescence staining. Control untreated semen was kept at 16 °C until it was processed.

These evaluations were also performed on semen normally sorted, as described in "Flow Sorting," and then 1) collected in 500 µL of 2% Tes-Tris-egg yolk buffer supplemented with 10 µL of frozen-thawed seminal plasma, centrifuged (800 x *g*) for 10 minutes, resuspended with 200 µL Androhep and 1% seminal plasma, and then kept at 16 °C for 2 hours (Group 1), as described by Grossfeld et al (2005), and 2) collected in 1 mL Tes-Tris-egg yolk buffer containing 10% of seminal plasma and then kept at 20 °C for 2 hours (Group 2), as described by Parrilla et al (2005).

Plasma Membrane Integrity— Sperm viability of the above-described groups was evaluated by incubating 50 µL of semen with propidium iodide (PI) and SYBR Green 14 (Molecular Probes, Eugene, Ore) at the final concentration of 23 µmol and 0.1 µmol respectively for 5 minutes at 37 °C in the dark under light-proof conditions.

Aliquots of the stained suspensions were placed on clean microscope slides and overlaid carefully

with coverslips; within 5 minutes at least 200 spermatozoa per sample were scored with the above-mentioned Nikon epifluorescence microscope. Spermatozoa stained with SYBR Green and not stained with PI were considered as viable. Spermatozoa both SYBR + and PI + and those SYBR – /PI + were considered as dead.

Statistical Analysis

Data are presented as mean ± SEM. All the experiments were repeated at least 6 times, and a minimum of 200 spermatozoa per slide were scored. The normal distribution of the data was checked using the Kolmogorov-Smirnov test. For the analysis of not normally distributed data (plasma membrane integrity), we used Kruskal-Wallis ANOVA and independent *t* test; the other data (HSP data) were analyzed using one-way ANOVA. The software used was Macintosh SPSS 11. The level of significance was set at $P < .05$.

Results

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Immunolocalization of Hsp60, Hsp70, Hsp90

In unsorted semen Hsp60 immunoreactivity was detected as little spots in the sperm midpiece, while a faint Hsp90 positive signal was present in the midpiece or throughout the sperm tail. No modification in both Hsp60 and Hsp90 immunoreactivity was recorded in sorted as compared to unsorted semen.

As for Hsp70, the great majority of unsorted spermatozoa (93 ± 1%) showed a strong triangular-shaped Hsp70 immunoreactivity in the middle of the equatorial segment of the sperm head (Figure 1A), whereas 5 ± 1% sperm cells exhibited different patterns: Hsp70 immunoreactivity in the equatorial line sometimes associated with a triangular signal, more rounded and fainter than in fresh semen, and/or a semicircular positive line on the anterior boundary of the equatorial segment; these patterns are typical of capacitated spermatozoa (Spinaci et al, 2005b) (Figure 1B). Very few spermatozoa (0.3% ± 1) displayed the fluorescence organized mainly in a thick subequatorial band with the triangular-shaped signal slightly rounded or flattened; this pattern is typical of spermatozoa that have undergone acrosome reaction owing to physiological (zona pellucida) and nonphysiological (calcium ionophore) inducers (reacted pattern) (Figure 1C). After the whole sorting procedure, the amount of spermatozoa exhibiting the different patterns changed (Figure 2); the percentage of spermatozoa with the triangular fluorescence on the equatorial segment (Hsp70 uncapacitated pattern) dramatically decreased to 24 ± 8%, whereas the percentage of sperm cells displaying the capacitated and reacted pattern increased to 65 ± 9 and 5 ± 2%, respectively.

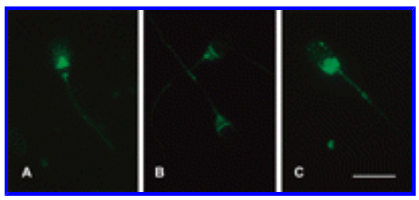

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Figure 1. Representative fluorescent micrographs of HSP70 immunolocalization in boar spermatozoa. **(A)** Freshly ejaculated sperm cell with the triangular immunoreactivity in the equatorial subsegment. **(B)** Spermatozoa displaying Hsp70 capacitated pattern; note the immunoreactivity in the equatorial line and the anterior boundary of the equatorial segment. **(C)** Sperm cell displaying Hsp70 reacted pattern, with the immunolabeling confined to a subequatorial band and a flattened triangular shaped area. Bar = 10 µm.

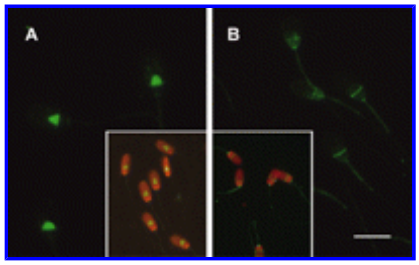

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Figure 2. Representative fluorescent micrographs of HSP70 immunolocalization in fresh and sorted boar spermatozoa. The inserts show Hsp70 green immunostaining revealed by FITC and red fluorescence staining of nuclei with PI. **(A)** Freshly ejaculated unsorted spermatozoa displaying Hsp70 uncapacitated pattern. **(B)** Sorted spermatozoa displaying Hsp70 capacitated pattern. Bar = 10 µm.

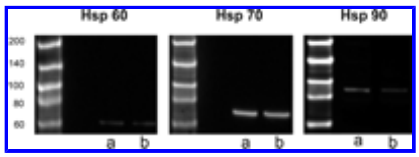

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Figure 3. Representative Western blots of Hsp60, Hsp70, and Hsp90 of control unsorted (lane a) and sorted (lane b) spermatozoa. Molecular weight standards (kDa) are on the left of each Western blot.

No label was detectable when the second antibody alone was used.

Western Blotting

Figure 3 shows a representative Western blot of Hsp60, Hsp70, and Hsp90; a single band of expected molecular weight was detected for Hsp60, Hsp70, and Hsp90, whose levels were detectable in all samples. The relative protein content (Hsp/ß-tubulin) is expressed in arbitrary units (AU). No significant differences between unsorted and sorted spermatozoa in the content of Hsp60 (6 ± 2 vs 5 ± 1 respectively), Hsp70 (89 ± 15 vs 99 ± 5 respectively), and Hsp90 (15 ± 3 vs 13 ± 4) were detected.

Effect of the Different Steps of the Sorting Procedure

Hsp70 Relocalization— Results are summarized in Figure 4. After staining procedure, a significant ($P < .001$) decrease in the percentage of spermatozoa displaying the triangular fluorescence on the equatorial segment (uncapacitated sperm) and a significant ($P < .05$) increase in the percentage of sperm cells with Hsp70 capacitated pattern were recorded. The passage through the flow cytometer, whether associated or not with the laser beam, did not induce any significant modification in the rate of the different Hsp70 patterns compared to Stained sperm. The All Sort sperm group, obtained after the whole sorting procedure deflecting live and dead cells, was characterized by a significantly lower percentage of spermatozoa with Hsp70 uncapacitated pattern and a higher percentage of Hsp70 capacitated pattern when compared with the unsorted ($P < .001$) and Stained ($P < .05$) groups (Figure 4).

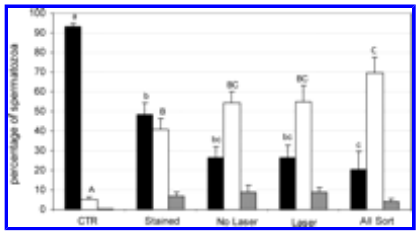

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Figure 4. Effect of sorting steps on the percentage of sperm cells displaying Hsp70 uncapacitated pattern (closed bar), Hsp70 capacitated pattern (open bar) and Hsp70 reacted pattern (striped bar). Different letters on the same type bars indicate a significant ($P < .05$) difference.

The results relative to the different protocols of collection and storage of sorted semen are presented in Figure 5. After collection, group 1 was characterized by a lower percentage of spermatozoa with Hsp70 uncapacitated pattern (21 ± 8%) and a higher percentage of Hsp70 capacitated pattern (68 ± 5%) than group 2 (67 ± 8 and 30 ± 4% respectively) ($P < .001$). The situation was reversed after 2 hours of storage, when Hsp70 uncapacitated pattern increased to around 65% in group 1 while decreasing to around 12% in group 2; the percentage of sperm cells displaying Hsp70 capacitated pattern, on the contrary, significantly lowered in group 1 (27 ± 5%) while significantly increasing in group 2 (87 ± 3%) ($P < .001$).

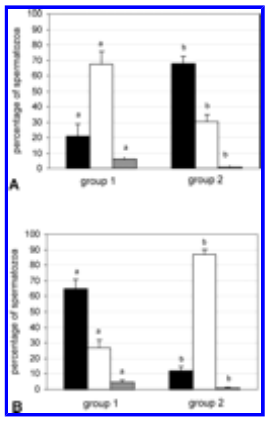

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Figure 5. Effect of collection **(A)** and 2 hours storage **(B)** protocols after sorting on the percentage of sperm cells displaying Hsp70 uncapacitated (closed bar), capacitated (open bar) and reacted (striped bar) pattern. Different letters on the same type bars indicate a significant ($P < .05$) difference.

No label was detectable when the second antibody alone was used.

Plasma Membrane Integrity— Results are summarized in Figures 6 and 7. Staining procedure induced a significant reduction in sperm viability as compared to control. The percentage of live spermatozoa was significantly ($P < .05$) lower in No Laser, Laser, and All Sort groups than in both unsorted and Stained spermatozoa (Figure 6).

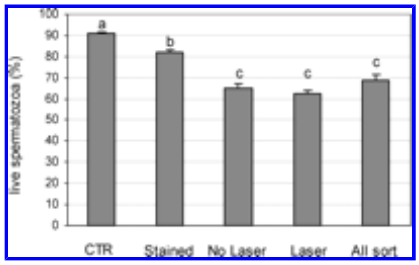

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Figure 6. Effect of sorting steps on boar sperm cell viability. Bars with different letters differ significantly ($P < .05$).

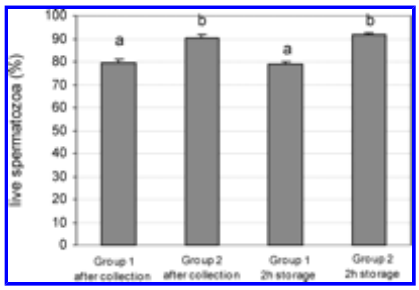

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Figure 7. Effect of collection and 2-hour storage protocols on boar sperm cell viability. Bars with different lettering differ significantly ($P < .05$).

The different collection protocols resulted in a significant difference in sperm viability, as group 2 showed the highest percentage of live cells (Figure 7). Sperm viability between the 2 groups remained significantly different after 2 hours of storage; the storage did not influence sperm viability within each group.

Discussion

In this study, the presence and the cellular distribution of Hsp60, Hsp70, and Hsp90 in fresh and sorted boar semen were investigated by immunofluorescence and Western blot. The effect of the different steps of sorting procedure on Hsp70 localization was also examined.

Hsp60 is localized in the sperm midpiece, possibly in mitochondria. Comparing sorted and control semen, no differences in either immunolocalization or Western blot were recorded. Hsp60 plays an important role as molecular chaperone during folding of newly formed mitochondrial proteins, but also during protein degradation aimed at eliminating proteins either folded incorrectly or aggregated as a result of cell stress (Ryan et al, 1997). As no Hsp60 consumption has been observed after the complete sorting process, the whole procedure shouldn't have induced any mitochondrial damage. These results are consistent with those from our previous study (Spinaci et al, 2005a), in which we observed that the great majority of sorted spermatozoa are characterized by high mitochondrial membrane potential, that is a good index of mitochondrial activity.

Hsp90 has been demonstrated to participate in regulating boar sperm motility by a mechanism similar to that of caffeine (Huang et al, 2000b). As for Hsp60, Hsp90 immunoreactivity and Western blot did not show any difference between control and sorted semen. Therefore, the sorting procedure does not seem to induce either quantitative modification or relocalization of this protein, which, on the contrary, has been reported to decrease, preceding a decline in motility, during cooling of boar spermatozoa (Huang et al, 1999).

As already reported (Spinaci et al, 2005b), Hsp70 immunoreactivity in boar fresh semen was observed on the equatorial segment membrane in a well-defined triangular-shaped area, named the equatorial subsegment (EqSS) by Ellis et al (2002). Most of the sorted spermatozoa showed a Hsp70 lateral migration within a range of patterns typical of capacitated sperm cells; in fact, during capacitation and, further on, acrosome reaction, Hsp70 undergoes redistribution and exposition on the sperm surface. Hsp70 represents an important binding site during porcine gamete interaction, particularly during the sperm-egg membrane fusion: the presence of anti-Hsp70 antibody in the fertilization medium has been demonstrated to reduce, in a concentration-dependent manner, the fertilization rate of both zona-intact and zona-free oocytes (Spinaci et al, 2005b).

Capacitation-like changes have been already demonstrated after sorting in boar spermatozoa (Maxwell and Johnson, 1997, 1999; Maxwell et al, 1996; Maxwell et al, 1998). Moreover, changes in motility have also been observed in flow-sorted boar spermatozoa, suggesting the beginning of the capacitation process (Parrilla et al, 2005). Those and our observations might indicate that the sorting procedure initiates plasma membrane reorganization which, if not stopped or controlled, could lead to capacitation and, further on, to acrosome reaction.

In order to optimize the single steps of sperm-sexing procedure by flow cytometry, it is important to focus the negative impact of the different stressors. We have therefore evaluated both Hsp70 redistribution, as a capacitation index, and the viability of spermatozoa progressively exposed to the sorting steps that may induce cell damage. As already recorded in a previous study (Spinaci et al, 2005a), following incubation with Hoechst, we observed a reduction of viable cells; a further decrease of viability was observed when spermatozoa were exposed to subsequent stressing events of sorting procedure, such as high pressure (No Laser group) associated with laser beam (Laser group) and charging and deflection of drops (All Sort group). However, as these last 3 groups did not differ in the percentage of viable cells, it seems likely that staining procedure and high pressure are the most injuring factors for sperm membrane, as already reported (Seidel and Garner, 2002; Garner and Suh, 2002; Suh et al, 2005; Garner, 2006).

The study of Hsp70 redistribution revealed that staining incubation destabilizes sperm membrane, increasing the number of cells that display Hsp70 capacitated pattern; even small temperature variations are known to strongly modify the physical state of membrane lipids, which are of critical importance for sperm membrane remodeling (Yanagimachi, 1994; Maxwell and Johnson, 1997). The mechanical stress induced by the passage of sperm through the flow cytometer seems to be only a weak stimulus for Hsp70 relocation, while the laser beam does not induce any further redistribution of the protein; this last finding is not surprising, as exposure of stained sperm to UV laser is very short (approximately 1 µsec) in the high-speed sperm sorter (Guthrie et al, 2002). The final steps of sperm sorting, charging, and deflection of drops seem to constitute an additional stimulus for Hsp70 relocation. However, the dilution in the collecting tube also seems to represent a stimulus for membrane remodeling, as collecting sorted semen directly onto a glass slide reduces the percentage of cells displaying Hsp70 capacitated pattern as compared with sorted semen collected in the catch fluid (data not shown).

When two different protocols for collection and storage of sorted spermatozoa were analyzed, the viability of group 1 semen, collected as described by Grossfeld et al (2005), was significantly lower when compared to group 2, collected as described by Parrilla et al (2005). However, it must be emphasized that the first group contained all sorted spermatozoa, live and dead, since it was obtained by centrifugation, which itself constitutes an injury, as demonstrated by Maxwell et al (1996); on the contrary, group 2 semen was collected, without centrifuging, directly from the bottom of the tube containing catch fluid, which is reached by sorted motile, therefore live, spermatozoa. This situation could explain the difference in the percentage of live sperm between the 2 groups, after both collection and storage.

Intriguing results on Hsp70 redistribution were obtained in these samples: in group 1 most spermatozoa displayed a Hsp70 capacitated pattern, while in group 2 the majority of sperm cells showed a triangular-shaped Hsp70 immunoreactivity typical of fresh uncapacitated semen. Therefore the collection method used by Parrilla et al (2005) on the basis of both viability and Hsp70 pattern seems to be the less injuring.

Surprisingly, after 2 hours of storage the situation was completely reversed: in fact, while most group 1 sperm cells went back to uncapacitated Hsp70 pattern, the great majority of spermatozoa in group 2 showed a membrane with capacitated Hsp70 character, even if the viability remained higher. These results may be due to the storage of group 2 sorted semen at 20 °C into the collection tube where motile spermatozoa, after having swum downward, remain in a fairly concentrated catch fluid. Therefore, using this storage protocol, sorted spermatozoa are probably allowed to undergo capacitation-like membrane remodeling. These results could explain the higher fertilization rate obtained by Parrilla et al (2005) by inseminating pig oocytes with sorted spermatozoa stored for 2 hours when compared with spermatozoa utilized just after sorting. Basing on these results, the choice of the storage protocol should be carefully considered.

Our results show that Hsp70 relocation could be reversed after 2 hours of storage in a small volume of medium in the presence of seminal plasma. This finding seems to be related, at least in part, to the seminal plasma ability to stabilize sperm membrane and reverse capacitation status with which boar spermatozoa emerge from the sperm sorter (Maxwell et al, 1996, 1998); in fact, storage of sorted spermatozoa in 200 µL of Androhep without seminal plasma did not induce the reversion of Hsp70 redistribution (data not shown). The reversibility of Hsp70 migration agrees well with the results obtained by Western blot that evidence a redistribution not associated with a consumption of the protein during membrane modifications induced by sorting process, as it occurs during in vitro sperm capacitation (Spinaci et al, 2005b).

In conclusion, our data demonstrate that the sperm sorting procedure, step by step, induces a redistribution of Hsp70, but not Hsp60 and Hsp90, in boar spermatozoa. Hsp70 relocation suggests a progressive remodeling of sexed sperm membrane towards capacitation-like changes. This finding agrees well with the observation that sexed spermatozoa do not require an additional maturation period for capacitation prior to IVF, as is needed for unsorted sperm (Rath et al, 1999); on the other hand, this artificial precapacitation is one cause of the well-known necessity to inseminate in vivo close to ovulation by depositing sexed semen near the site of fertilization (Johnson et al, 2005). The possibility of reversing Hsp70 relocation by storing sorted spermatozoa, after centrifugation, in a small volume in the presence of seminal plasma is therefore important for preserving sperm membrane by premature relocation and exposition on the outer leaflet of Hsp70, a protein that plays a role during sperm-oocyte membrane interaction.

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Footnotes

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