Impact of Storage and Purification on Mitochondrial Membrane Potential of Boar Spermatozoa

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Abstract
This study aimed to evaluate the effect of semen purification and storage on sperm mitochondrial membrane potential ($\Delta \Psi_m$). Gel-free whole ejaculates were collected from five proven fertile Large White boars aged two to three years. Aliquots of fresh semen were split, diluted in one step with commercial extenders and incubated at 37°C for 5-10 minutes. Semen was cooled to 18°C and packaged in 15-ml sterile propylene tubes. After 4-10 hours post-semen collection, stored semen was purified by colloidal centrifugation. After 48 hours post-semen collection, stored semen was incubated at 37°C and evaluated after 45 minutes for motility, velocity and sperm $\Delta \Psi_m$. Samples were stained with 2.99 $\mu$M JC-1 and 2.32 $\mu$M EH-1 and assessed by Fluorescence microscopy. After centrifugation a significant improvement of motility ($P<0.035$), and velocity ($P<0.012$) was noticed. The percentage of spermatozoa with intact plasma membrane and high/low mitochondrial membrane potential was statistical higher after centrifugation and storage at 18°C for 48 hours. In conclusion, colloidal purification of boar semen can improve sperm quality and mitochondrial membrane potential.

Keywords: boar, mitochondrial membrane potential, purification, sperm storage

1. Introduction
Fertility of boar spermatozoa decreases with increase in storage time during liquid preservation. During storage, the boar spermatozoa undergo several changes including diminished motility, viability and alterations in membrane permeability. Boar sperm seems to be sensitive to peroxidative damage due to the relative high content of polyunsaturated fatty acids in the phospholipids of the membrane [1] and the relative low antioxidant capacity of boar seminal plasma [2]. Excessive ROS formation by spermatozoa during preservation has been associated with a decrease in the function of spermatozoa during preservation [3]. The consequences of lipid peroxidation are membrane damage, inhibition of respiration and leakage of intracellular enzymes [4]. When the lipid peroxidation cascade is stimulated, 60% of fatty acid is lost from the membrane [5], resulting in loss of membrane integrity. Oxidative damage to mitochondrial membrane architecture may be a factor of major important to explain the impaired fertility and motility of preserved spermatozoa [6]. Mitochondria, the sites of oxidative phosphorylation (OXPHOS), are located in the sperm mid-piece at the extreme anterior end of the flagellum. As in other cells, sperm mitochondria produce ATP through aerobic respiration that is typically required for a cell to survive. Tests for mitochondrial function are usually highly correlated with viability assays [7; 8]. Mitochondrial respiration would be the predominant source of ATP required for flagellar movements which is the greatest energy consumption for the sperm cell. Therefore, sperm
motility would indirectly reflect the ability of mitochondria to propel the sperm and represent a way to detect the ATP production. The introduction of fluorescent probes such as JC-1, that stain specific sperm compartments, has enhanced the ability of researchers and clinicians to evaluate the activity of mitochondria. Since any changes in mitochondrial function may be reflected in sperm motility [9] and the proportion of 5,5′; 6,6′-tetrachloro-1,1′, 3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1)-stained spermatozoa are significantly correlated with motility [10] mitochondrial activity of boar spermatozoa can be determined with JC-1, a mitochondria specific stain with a high sensitivity. JC-1 is a lipophilic cationic fluorescent carbocyanine dye that is internalized by all functioning mitochondria, where it fluoresces green. In highly functional mitochondria, the concentration of JC-1 inside the mitochondria increases and the stain forms aggregates that fluoresce orange. When human spermatozoa were divided into high, moderate and low mitochondrial potential groups, based on JC-1 fluorescence, the in vitro fertilization rates were higher in the high potential group than in the low potential group [7].

However, changes in the mitochondrial membrane potential of boar spermatozoa during liquid preservation have not been studied in detail. The aim of the present study was to evaluate the effect of semen purification and storage on sperm mitochondrial membrane potential ($\Delta \Psi_m$).

2. Materials and methods

Reagents

The 5,5′A, 6,6A-tetrachloro-1,1′,3,3′-A-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) (218615) was obtained from Santa Cruz Biotechnology Inc, USA. Ethidium homodimer (EH-1) (46043) was obtained from Sigma-Aldrich CO, (St. Louis, MO). Semen extender INRA 96 was obtained from IMV Technologies, France. PorciPure® colloid was obtained from Nidacon International AB, Göthenborg, Sweden.

Semen collection

Semen was collected from five mature fertile Large White boars (2–3 years old) of proven fertility from a pig farm in Pieria in North Greece. Five ejaculates each from five boars were collected by gloved hand technique with the help of dummy sow the sperm-rich fraction was collected in a pre-warmed thermos, while the gel fraction was held on a gauze tissue covering the thermos opening. After collection, semen characteristics (volume, sperm concentration and subjective sperm motility) were microscopically evaluated using standard laboratory techniques. Only gel-free whole ejaculates of $\geq 0.2 \times 10^9$ sperm/ml and 75% sperm progressive motility were used. The semen then was extended with commercial extenders and transported to the laboratory. Once in the laboratory, the semen samples were incubated at 37°C until analyzed.

Semen analysis

Sperm concentration of fresh semen was measured by a precalibrated photometer (SpermaCue, Minitub-Germany). In preserved samples, 1 μl of semen was diluted with 99 μl of 3% NaCl solution containing 0.02% eosin and sperm cell concentration was estimated using an improved Neubauer hemocytometer chamber.

Sperm motility was assessed in stored semen after reactivating in a water bath at 35°C for 30 min before examination. Sperm motility was evaluated by keeping a drop of semen on a pre-warmed slide and estimating the percentage of sperm possessing progressive motility at $\times 1000$ magnification using a phase contrast microscope (Leica DMLB 2000, Germany) equipped with a heating stage (35°C). The motility was expressed as percentage of progressively motile spermatozoa. The speed of sperm motility was scored on a scale from 1 to 4 (slowest to fastest, respectively).

For sperm agglutination evaluation, a 10-μL aliquot of each sample was placed onto a pre-warmed glass slide, covered with a warm glass cover slip, and observed at magnification $\times 125$ under a phase-contrast microscope (Leica DMLB 2000, Germany) equipped with a warm stage at 37°C. Approximately 50 motile sperm cells were visually examined in each of four fields to estimate the percentage of head-to-head agglutinated sperm, which was defined as the number of sperm agglutinated to at least one other sperm per total motile sperm.

Preparation of fluorescent probes

Preparation of 3-mM stock solution of JC-1

The 5-mg vial content (0.005 g) was dissolved in 2.55 ml of DMSO (1.96 mg dye/ml). Aliquots of...
0.1 ml stock solution were transferred in eppendorf tubes and stored at -20°C until used.

**Preparation of 1.167-mM stock solution of EH-1**

The 1-mg vial content (0.001 g) was dissolved in 1 ml DMSO (1 mg dye/ml; 1 μg dye/μl). Aliquots of 0.1 ml stock solution were transferred in eppendorf tubes and stored at -20°C until used.

**Assessment of Mitochondrial Membrane Potential**

All staining procedures were carried out at room temperature (22-25°C). Semen samples were extended to a concentration of 30-100x10⁶ sperm/ml. A 1 μl of EH-1 stock solution and 0.5 μl of the stock solution were transferred in a light-protected tube containing 500 μl of sperm suspension. The stains were mixed well with sperm suspension to achieve final dye concentrations of 1.96 μg JC-1/ml (2.99 μM) and 1.99 μg EH-1/ml (2.32 μM). Stained spermatozoa were incubated at 37°C for 30 minutes before analyses. A 10-μl sample of stained spermatozoa suspension was placed on a slide, cover slipped and evaluated immediately by fluorescent microscope (LEICA DMLB, Germany) at 1000x magnification. Spermatozoa stained with JC-1 display either green fluorescence for mitochondria with low to medium membrane potential, or orange fluorescence for mitochondria with high membrane potential in the tail [10]. An average of 200 cells was evaluated for each sample.

**Experimental design**

Ejaculates (n= 5) were collected from five boars (1 ejaculate/boar) and incubated at 37°C until initial assessment of fresh semen. Aliquots of fresh semen were split, diluted in one step (30-40x10⁶ sperm/ml) with commercial extenders and incubated at the same temperature for 5-10 minutes. Semen was cooled to 18°C (~0.10°C/minute) and packaged-stored in 15-ml sterile propylene tubes. The air-filled spaces above semen were less than 4.5% of the tube’s capacity. Semen tubes were placed horizontally in a cooling box and rotated at least twice a day throughout the whole storage period.

After 4-10 hours post-semen collection, stored semen was purified by PorciPure® (Nidacon International AB, Göthenborg, Sweden) colloidal centrifugation. Four ml of the colloid were transferred to a sterile conical centrifuge tube. One ml of diluted semen was layered on the top of the colloid column and centrifuged at 300 x g for 20 minutes at RT using a swing-out rotor. All the fluid above sperm pellet was aspirated. Sperm pellet was transferred to a new tube and was resuspended in 1 ml of INRA-96 medium (IMV Technologies, France). After 48 hours post-semen collection, stored semen was incubated at 37°C and evaluated after 45 minutes for endpoints described above.

**Statistical analysis**

Data were presented as means ± SEM and analyzed using independent t-test and one-way ANOVA followed by Duncan’ multiple range test.

### 3. Results

The effect of colloidal purification of boar semen on sperm kinetic activity and incidence of sperm agglutination are shown in table 1. Before and after purification of boar semen no significant difference on sperm total motility was noticed. However, a significant difference on sperm progressive motility (P<0.035), velocity (P<0.012) and agglutination (P<0.006) was observed.

#### Table 1. Effect of colloidal purification of boar semen on sperm kinetic activity and incidence of sperm agglutination

<table>
<thead>
<tr>
<th>Sperm</th>
<th>Semen</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before purification</td>
<td>After purification</td>
<td></td>
</tr>
<tr>
<td>Total motility (%)</td>
<td>72.0±3.8</td>
<td>86.0±5.6</td>
</tr>
<tr>
<td>Progressive motility (%)</td>
<td>63.0±3.4</td>
<td>80.0±5.5</td>
</tr>
<tr>
<td>Velocity grade (0-4)</td>
<td>1.4±0.2</td>
<td>2.6±0.3</td>
</tr>
<tr>
<td>Agglutination grade (0-5)</td>
<td>1.9±0.4</td>
<td>0.0±0.0</td>
</tr>
</tbody>
</table>

*Significant effect for semen purification on mean (± SEM) values of five ejaculates collected from five different boars. Each ejaculate was purified after 4-10 hours of storage at 18°C.

In figure 1 four groups of sperm population were observed after using JC-1 assay: (i) sperm cells with intact plasma membrane and high ΔΨm (IPM+HMP), (ii) cells with intact plasma membrane and low ΔΨm (IPM+LMP), (iii) cells with intact plasma membrane and high/low ΔΨm (IPM+HLP) and (iv) cells with damaged plasma membrane and low ΔΨm (IPM+LMP). After
purification there was a significant decrease in the sperm cells with high and low $\Delta \Psi_m$, while a significant increase (99.8%) in the sperm cells with high/low $\Delta \Psi_m$ was noticed.

Storage of boar semen at 18°C for 48 hours had no influence on sperm kinetic activity and incidence of sperm agglutination compared to the storage for 4-10 hours (Table 2).

<table>
<thead>
<tr>
<th>Sperm Semen storage duration</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total motility (%)</td>
<td></td>
</tr>
<tr>
<td>4-10 hours</td>
<td>72.0±3.7</td>
</tr>
<tr>
<td>Progressive motility (%)</td>
<td></td>
</tr>
<tr>
<td>4-10 hours</td>
<td>63.0±3.4</td>
</tr>
<tr>
<td>Velocity grade (0-4)</td>
<td></td>
</tr>
<tr>
<td>4-10 hours</td>
<td>1.4±0.2</td>
</tr>
<tr>
<td>Agglutination grade (0-5)</td>
<td></td>
</tr>
<tr>
<td>4-10 hours</td>
<td>1.9±0.4</td>
</tr>
</tbody>
</table>

4. Discussion

An important test for determining semen quality may be the assessment of membrane mitochondrial potential ($\Delta \Psi_m$) in intact spermatozoa since mitochondrial function has been related to sperm motility. JC-1 appears to be the best probe for the detection of $\Delta \Psi_m$ changes in spermatozoa and to predict sperm fertility potential.

The results of this study show a significant correlation between the capacity of the $\Delta \Psi_m$ and the parameters of the routine semen analysis.
Among those showing the strong correlation of ΔΨm with progressive motility and the velocity which enhances previous results [11, 7, 8 and 12]. Our results showed an increase in capacity of the mitochondrial membrane (ΔΨm) of spermatozoa after purification of boar semen and its storage at 18°C for 48 hours. This increase indicates good mitochondrial function, which leads to renewal of ATP. The energy produced by increasing the production of ATP may be responsible for the improvement observed in the progressive motility and velocity of sperm [13]. In contrast, a reduction of capacity of the mitochondrial membrane potential of spermatozoa was found in boar semen preserved at 18 °C for 0, 24, 48, 72 and 96 hours without prior colloid centrifugation [14]. It has been reported by the authors of the above findings that this reduction may result in dysfunction of mitochondria resulting in non-renewal of ATP. Also, it has been found that the integrity of mitochondria during programmed cell death may be affected by the release of apoptotic agents present in the inner and outer mitochondrial membrane [15]. The effect of colloid centrifugation in selecting porcine sperm with higher motility has been well documented [16-19]. However, other studies have not detected significant differences. This is probably related to the high variability that could be present in some boars, differences in the colloid density procedures [20, 21] or differences in the system of measurement. However, the differences in the motility reported in this study showed that the colloid is important to optimize recovery of a higher percentage of spermatozoa with a high velocity which could be related to the capacitation process due to a significant increase of tyrosine phosphorylation and calcium intake [22]. This sperm subpopulation with high velocity could be related to the fertilizing capacity [23, 24]. The study reported here clearly demonstrates that boar spermatozoa exhibit an enhanced function after a combination of washing and colloid centrifugation. These results support the extensive data from human studies [25-29].

5. Conclusions

In conclusion, colloid purification improves the quality of boar semen. Both purification and storage had an influence on mitochondrial membrane potential.
References


