Effect of freezing and thawing rates on the post-thaw viability of boar spermatozoa frozen in FlatPacks and Maxi-straws

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Abstract

The effects of different freezing and thawing rates on the post-thaw motility and membrane integrity of boar spermatozoa, processed as split samples in Maxi-straws or flat PET-plastic packages (FlatPack) were studied. A programmable freezing device was used to obtain freezing rates of either 20, 50 or 80 °C/min. Thawing of the samples was performed in a bath of circulating water; for 40 s at 50 °C or 27 s at 70 °C for Maxi-straws and 23 s at 35 °C, 13 s at 50 °C or 8 s at 70 °C for the FlatPacks. Sperm motility was assessed both visually and with a computer assisted semen analysis (CASA) apparatus, while plasma membrane integrity was assessed using the fluorescent probes Calcein AM and ethidium homodimer-1. Temperature changes during freezing and thawing were monitored in both forms of packaging. Values for motile spermatozoa, sperm velocity and lateral head displacement variables were significantly ($p<0.05$) higher for samples frozen in FlatPacks than in Maxi-straws, with superior results at higher thawing rates. Freezing at 50 °C/min yielded better motility than 20 or 80 °C/min, although the effect was rather small. Neither freezing rate nor thawing rate had any effect on membrane integrity ($p>0.05$). A significant boar effect was seen for several parameters. The most striking difference in temperature courses between containers was a 4–5-fold lowering of the thawing rate, between −20 and 0 °C, in the center of the Maxi-straw, compared with the FlatPack. This is apparently due to the insulating effect of the thawed water in the periphery of the Maxi-straw. The improvement in sperm motility seen when using the FlatPack appears to be related to the rapid thawing throughout the sample, which decreases the risk of cell damage due to recrystallization during thawing. Since sperm motility patterns have been reported to be correlated with fertility both in vitro and in vivo it is speculated that the use of the FlatPack might improve the results when using frozen-thawed boar spermatozoa for artificial insemination.

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1. Introduction

Frozen boar semen can be used as an alternative to the shipping of live animals to export valuable genetic material. It not only reduces transportation costs but it is optimal from a herd health viewpoint. Use of cryopreserved boar semen for artificial insemination (AI) has, however, been shown to be a more delicate procedure than for some other species, e.g. cattle or dogs. In pigs, a distinct reduction in farrowing rate and litter size has been found with cryopreserved semen, when compared to fresh spermatozoa (Johnson, 1998). Another drawback of frozen boar spermatozoa for AI is the large volume needed for one insemination dose. The commercially most widely used package nowadays (Almlid and Hofmo, 1996), the 5 ml Maxi-straw, precludes a rapid freezing and thawing because of its cryobiologically unsuitable shape (5.4 mm i.d.). During freezing — and even more so during thawing — substantial differences between the temperature courses in the center and in the periphery of the straw (Weitze et al., 1987) occur during water-phase changes. To circumvent these problems, several authors have frozen boar spermatozoa in other, more cryologically suited packages, 0.25 ml Maxi-straws, 0.5 ml Medium-straws and different flat packages (Bwanga et al., 1990; Berger and Fischerleitner, 1992; Simmet, 1993). Although improvements in in vitro sperm viability have been seen, these packages are still not well adapted for use in practice. Either several packages are needed for one AI-dose (e.g. Maxi- or Medium-straws) or there is no convenient system for storing the frozen semen (e.g. bags).

We have therefore developed a new flat plastic package, the FlatPack (FP) for freezing boar semen, which allows a quick and more uniform freezing and thawing than the Maxi-straw. As the FlatPack holds 5 ml, there is room for a single AI-dose in one pack. It also fits into any conventional Dewar canister, thus making it convenient for commercial use.

Freezing and thawing rates have been shown to influence the post-thaw survival of a number of cells (Mazur, 1985). This also applies to spermatozoa from various species, although optimum cooling rates for semen of domestic animals are generally considered to range quite widely, 10–100°C/min (Watson, 1990). The aim of the present study was to ascertain the effect of a limited number of freezing and thawing rates on post-thaw motility and plasma membrane integrity when semen was frozen in Maxi-straws or FlatPacks.

2. Materials and methods

2.1. Semen freezing protocol

Semen from four Swedish Yorkshire boars aged between 10 and 31 months, having proven fertility with fresh semen and showing a minimum of 70% motile and 80% morphologically normal spermatozoa, was collected with the gloved-hand technique and frozen once weekly. Semen was processed according to the method described by Westendorf et al. (1975) and modified by Bwanga et al. (1990). In brief, the sperm-rich fraction was collected in an insulated thermos flask, separating the gel fraction through a gauze during collection. Semen was diluted in 250 ml centrifuge flasks (1+1 to 1+3) with BTS (Pursel and Johnson, 1975), with added penicillin and streptomycin, at 32°C, giving a volume of 200–220 ml. Sperm concentration (Bürker chamber) and motility (phase-contrast microscope) were then deter-
mined. The diluted semen was allowed to stand in a cooling centrifuge (Centra MP4R, IEC), set at +15°C, for 3 h, after which it was centrifuged at 800×g for 10 min. The supernatant was discarded, and the volume and concentration (Bürker chamber) was measured. The remaining semen was rediluted with a second extender (Ext.II, 80 ml 11% lactose+20 ml egg yolk) at a ratio of one to two parts semen to one part extender. After thorough mixing, the semen was further cooled to +5°C for 2 h in the centrifuge. At this temperature, the semen was diluted slowly with a third extender, consisting of 89.5 ml Ext.II, 9 ml glycerol and 1.5 ml Equex STM (Nova Chemicals Sales, Inc., Scituate, MA, USA, equivalent to Orvus Es Paste, Graham et al., 1971); two parts semen to one part extender, giving a 3% glycerol final concentration. The final sperm concentration was 1×10^9 spz/ml, which was checked in a Bürker chamber. The work at +15°C and +5°C was done in a cooled cabinet (IMV, France). After mixing, the extended semen was split into Maxi-straws, sealed with sealing balls (Minitüb, Germany), or in flat plastic containers, both containers having a volume of 5 ml, and frozen. The FlatPack was made of polyethylene terephthalate (PET) plastic, with a thickness of 0.2 mm. Its dimensions (length: 30 cm, width: 22 mm) leave the semen in a thin film (280 mm×18 mm×1 mm). The FlatPack, presealed on three sides, was filled through a long flat catheter which reached the bottom and was withdrawn during filling. The PET-package was finally end-sealed with a thermo-sealer (Auto-Seal 102, Nitech AB, Sweden). Three Maxi-straws and four FlatPacks were frozen from each boar on every freezing occasion. Both packages were put horizontally on a separate rack, and the air bubble in the Maxi-straw was centered to the middle. The doses were transferred to a programmable freezing device (Mini Digitcool 1400, IMV, France), which was set at +5°C. The freezing programme had the following steps: from +5 to −6°C with 3°C/min, 1 min holding at −6°C, from −6 to −140°C with different freezing rates; 20, 50 or 80°C/min. The samples were then taken from the freezing chamber and plunged into liquid nitrogen (LN₂, −196°C). The sequence of freezing rates was rotated each week, for 12 consecutive weeks, making four replicates of each freezing rate. After 2–5 days of storage, the samples were removed from the LN₂, and thawed as follows: Maxi-straw: 40 s in a 50°C circulating waterbath or 27 s at 70°C, FlatPacks: 23 s at 35°C, 13 s at 50°C or 8 s at 70°C. The packages were dried carefully with a paper-towel before opening and the semen was examined further.

2.2. Temperature measurements during freezing and thawing

In representative groups of Maxi-straws and FlatPacks the temperature was measured (Fig. 1) with a type K copper-constantan thermocouple during freezing and thawing. One thermocouple was held in the center and one in the periphery of the Maxi-straw. In the FlatPack, two thermocouples were located in the centre, i.e. without touching the package wall, about 1 cm apart. The tips of the thermocouples were placed 3–6 cm from the end of the Maxi-straws or FlatPack (Fig. 2) by means of plastic cushions, when they were being filled prior to freezing. All thermocouples were connected to one temperature recorder (Chessel® Model 4180M, Chessel Ltd., Worthing, West Sussex, England) during freezing and to another (Chessel Model 324A, Chessel Ltd., Worthing, West Sussex, England) during thawing. The temperature changes in the semen were recorded repeatedly and from the printouts the freezing (Fig. 1A) and thawing (Fig. 1B) processes were calculated (Table 1).
Fig. 1. Temperature changes during freezing (A) and thawing (B) in 50°C water bath for boar semen frozen in Maxi-straw or FlatPack. (A) Thin lines: FlatPack; thick lines: Maxi-straw; unbroken lines: 20°C/min; broken lines: 50°C/min; dotted lines: 80°C/min. (B) Unbroken line: Maxi-straw (central position); broken line: Maxi-straw (peripheral position); dotted line: FlatPack.
Fig. 2. Freezing packages used; Maxi-straws (upper) and FlatPacks (lower).

### Table 1

Some characteristics of the temperature course during freezing (A) and thawing (B) measured in semen frozen in Maxi-straws or FlatPacks

<table>
<thead>
<tr>
<th>Type of package</th>
<th>Freezing rate in chamber (°C/min)</th>
<th>Freezing rate, in packagea</th>
<th>Duration of freezing point plateau (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maxi-straw</td>
<td>20</td>
<td>34</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>65</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>92</td>
<td>45</td>
</tr>
<tr>
<td>FlatPack</td>
<td>20</td>
<td>28</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>65</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>92</td>
<td>20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Type of package</th>
<th>Thawing temperature (°C)</th>
<th>Location in the straw</th>
<th>Thawing rateb (°C/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maxi-straw</td>
<td>50</td>
<td>Center</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Periphery</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>Center</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Periphery</td>
<td>3.4</td>
</tr>
<tr>
<td>FlatPack</td>
<td>35</td>
<td>–</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>–</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>–</td>
<td>5.0</td>
</tr>
</tbody>
</table>

\(a\) From \(-5\) to \(-60\)°C, after freezing point plateau.

\(b\) From \(-20\) to \(0\)°C.
2.3. Assessment of sperm viability post-thaw

After thawing, the semen was diluted slowly at room temperature (20–25°C), 1:20 (0.25+4.75 ml) with an extender consisting of 95 ml BTS and 5 ml Ext.II (lactose/egg-yolk solution). The addition of Ext.III prevented the spermatozoa from sticking to the glassware used during motility analysis. The sample was divided so that one 0.5 ml aliquot was used for assessment of membrane integrity, and 4.5 ml for motility analysis.

2.3.1. Sperm motility

The diluted semen was held in a water-bath at 38°C for 20 min before analysis. The sperm concentration used for motility analysis was about 50×10^9 spz/ml. Motility was measured both visually (phase contrast microscopy) and by means of a CASA apparatus, the Strömberg-Mika-CMA Windows version 1.1 (MTM Medical Technologies, Montreux, Switzerland). The setting parameters for the SM-CMA program were: 32 frames in which a spermatozoa had to be present in at least 16 in order to be counted, time resolution=20 ms (50 Hz), an object with a velocity (VAP)<10 μm/s was considered immotile and objects with a velocity >25 μm/s were deemed motile. Spermatozoa deviating <10% from a straight line were designated linear motile spermatozoa, and those having a radius <25 μm were classified as circular motile. After incubation and mixing in the tube, 5 μl of semen were placed into a 10 μm deep Makler counting chamber (Sefi Medical Instruments, Israel). The sample was inserted in the microscope and each time eight predestinated fields were recorded for 15 s (total 2 min) and the analysis was made from a video tape on a later occasion. The visual motility assessment (% motile spermatozoa, ‘Motsubj’) was made directly. The following motility parameters were used: ‘MotCMA’, % motile spermatozoa with CASA; ‘Circle’, % spermatozoa with circular motility; ‘VSL’, straight linear velocity (μm/s); ‘VAP’, average path velocity (μm/s); ‘VCL’, curvilinear velocity (μm/s); ‘LHD’, lateral head displacement (μm); ‘BCF’, beat cross frequency (n/s).

2.3.2. Sperm membrane integrity

Assessment was made by incubating the frozen-thawed spermatozoa in a staining medium containing the membrane-permeant cytoplasmic esteras marker Calcein AM (CAM) and the membrane-impermeant DNA-marker ethidium homodimer (EthD-1) (Live/Dead™ Viability/Cytotoxicity kit, Molecular Probes Inc., Oregon, USA). Stock solutions of CAM and EthD-1 were diluted in DMSO, giving concentrations of 1.005 and 1.167 mM, respectively. The staining medium was prepared by mixing 4 μl of EthD-1 (stock solution) with 1 ml PBS and vortexing well for 1 min. 10 μl of CAM (stock solution) was then added before vortexing for 1 min. In the staining medium, the concentration of CAM was 10.05 μM and of EthD-1, 4.67 μM. The stock and the staining solutions were kept in the dark and stored in the freezer (−20°C) until use. 20 μl of semen and 20 μl of staining medium were mixed and incubated in the dark for 15–30 min at 33–35°C. Random fields were observed under 600 magnification with epifluorescence illumination (Fig. 3) on the warm stage (37°C) of a Laborlux-11 Leitz microscope using a 470–490 nm excitation filter, a 510 dichroic beam splitter and a 520 nm barrier filter. One hundred spermatozoa were examined in each of two 5 μl aliquots from a stained sample. Three staining patterns could be discerned: (a) ‘live’, having an intact plasmalemma when entirely green with CAM, and unstained with EthD-1;
Fig. 3. Fluorescence staining patterns of boar spermatozoa using Calcein AM (CAM) and ethidium homodimer-1 (EthD-1). (A) ‘live’, having an intact plasmalemma stained entirely green with CAM, and unstained with EthD-1; (B) ‘moribund’, with the acrosome stained green with CAM, but the post-acrosomal region stained red with EthD-1, and (C) ‘dead’, having both a damaged plasmalemma and acrosomal membrane when the cells were unstained with CAM, but stained red with EthD-1.

(b) ‘moribund’, with the acrosome stained green with CAM, but the post-acrosomal region stained red with EthD-1; and (c) ‘dead’, having both a damaged plasmalemma and acrosomal membrane when the cells were unstained with CAM, but stained red with EthD-1. Only a sub-population of the spermatozoa belonging to category a were motile, but since they became immotile within a few seconds of illumination, no notice was taken as to whether the spermatozoa were motile or not. All green spermatozoa were therefore deemed viable by this test.

2.3.3. Statistical analysis

Data (as mean values) for post-thaw motility and plasma membrane integrity were analyzed by analysis of variance (ANOVA) using the general linear model (GLM) procedure from the Statistical Analysis Systems software (SAS Institute Inc., Cary, NC, USA, 1994). The statistical model used included the effects of boar, freezing rate, thawing rate, and the interaction between the freezing and thawing rates. A significance level of $p<0.05$ was applied.

3. Results

3.1. Temperature measurements during freezing and thawing

The only real difference noticed between packages during freezing was a longer freezing point plateau (FPP) for the Maxi-straws, and for both types of package the FPP increased at a slower freezing rate. There was a difference in the length of the FPP when measuring at different locations within the freezing packages. This difference was most pronounced in the Maxi-straw (longer FPP in the center versus periphery), but was also noted to a lesser extent in the FlatPack. After the FPP was finished, the freezing rates (between $-5$ and $-60^\circ C$) of both package forms were practically identical (Table 1A, Fig. 1A). On the other
hand, there were remarkable differences in the course of thawing, in the interval of −20 to 0°C, both when comparing the two packages, and even within the Maxi-straw. In this temperature interval, the thawing rate was about 4–5 fold higher in the FlatPack than in the center of the Maxi-straw, and 3–4 times faster in the periphery than in the center of the Maxi-straw (Table 1, Fig. 1B). Due to the very quick course of thawing in the FlatPack, the temperature difference that could be measured between different locations in this package was negligible.

3.2. Sperm viability post-thaw

3.2.1. Plasma membrane integrity

Neither freezing rate nor thawing regime had any significant influence on plasma membrane integrity (Table 2), although there was a tendency to improvement when thawing at 70°C for both Maxi-straw and FlatPack. There was however a statistically significant boar effect \((p<0.05)\) on this parameter (Table 2). The values of membrane intact spermatozoa were constantly higher than those of subjective motility, with a 20%-point difference for Maxi-straws and about 10%-points for FlatPacks.

3.2.2. Motility measurements

3.2.2.1. Percent motile spermatozoa. There was a significant effect on the number of motile spermatozoa, whether estimated visually or with CASA, by freezing rate, thawing regime and boar (Table 2). Temperatures of 50°C/min gave better results than 20

Table 2
Effects of freezing rate, thawing rate, and boar on percent spermatozoa with intact plasma membranes and percent motile spermatozoa (means) of frozen-thawed boar spermatozoa

<table>
<thead>
<tr>
<th>Variable</th>
<th>Label</th>
<th>n</th>
<th>Intact plasma membranes (%)</th>
<th>Motility CASA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freezing rate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20 80</td>
<td>57.7 a</td>
<td>44.4 a</td>
<td>51.5 a</td>
</tr>
<tr>
<td></td>
<td>50 80</td>
<td>59.7 a</td>
<td>47.2 b</td>
<td>54.6 b</td>
</tr>
<tr>
<td></td>
<td>80 80</td>
<td>58.6 a</td>
<td>42.6 c</td>
<td>50.0 c</td>
</tr>
<tr>
<td>Thawing rate&lt;sup&gt;b&lt;/sup&gt;</td>
<td>M50 48</td>
<td>57.5 a</td>
<td>37.9 a</td>
<td>43.2 a</td>
</tr>
<tr>
<td></td>
<td>M70 48</td>
<td>59.7 a</td>
<td>40.3 b</td>
<td>45.7 a</td>
</tr>
<tr>
<td></td>
<td>FP50 48</td>
<td>57.9 a</td>
<td>45.5 c</td>
<td>54.6 b</td>
</tr>
<tr>
<td></td>
<td>FP70 48</td>
<td>58.2 a</td>
<td>49.5 d</td>
<td>58.8 c</td>
</tr>
<tr>
<td></td>
<td>FP70 48</td>
<td>60.0 a</td>
<td>50.5 d</td>
<td>58.0 c</td>
</tr>
<tr>
<td>Boar</td>
<td>1 60</td>
<td>56.7 a</td>
<td>44.8 a</td>
<td>52.8 a</td>
</tr>
<tr>
<td></td>
<td>2 60</td>
<td>55.3 a</td>
<td>39.8 b</td>
<td>47.3 b</td>
</tr>
<tr>
<td></td>
<td>3 60</td>
<td>59.3 b</td>
<td>47.2 c</td>
<td>54.4 a</td>
</tr>
<tr>
<td></td>
<td>4 60</td>
<td>63.3 c</td>
<td>47.2 c</td>
<td>53.7 a</td>
</tr>
<tr>
<td>Residual SD&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.2</td>
<td>5.8</td>
<td>8.1</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Values with different letters within column and variable differ significantly \((p<0.05)\).
<sup>b</sup> °C/min in freezing chamber.
<sup>c</sup> M: Maxi-straw, FP: Flat-pack; 35, 50 and 70: temperature of thawing bath (°C).
<sup>d</sup> After adjustment for the effects included in the model.
<sup>e</sup> Visual motility %.
or 80°C/min. For the different thawing regimes the FlatPack yielded significantly better post-thaw motility than did the Maxi-straw. When comparing within-package there was an improvement in motility on using higher temperature in the thawing bath. In the Maxi-straw, this difference was only significant \( (p<0.05) \) for subjective motility. However, in the Flat-Pack there were significantly more motile spermatozoa when thawing at 50°C than at 35°C. In contrast to the Maxi-straw, no further improvement in motility could be seen for the FlatPack by increasing the thawing temperature from 50 to 70°C.

3.2.2.2. Linear and circular motile spermatozoa. Freezing rate, thawing regime and boar all had a statistically significant effect on the proportion of linear motile spermatozoa, whereas only thawing rate and boar significantly influenced the proportion of circular motile spermatozoa \( (p<0.05) \) (Table 3). More spermatozoa displayed linear motility in the samples frozen in FlatPacks and thawed at 50 and 70°C, than in Maxi-straws frozen at both 50 and 70°C, as well as in FlatPacks thawed at 35°C. The FlatPack samples thawed at 50 and 70°C contained a significantly smaller proportion circular motile spermatozoa, than did the other thawing regimes (Table 3).

3.2.2.3. Sperm velocity and lateral head displacement. The FlatPack gave a significantly higher sperm velocity post-thaw than did the Maxi-straw. A very similar picture was seen for VSL, VAP and VCL. Therefore only VAP is shown in Table 3. Sperm velocity was increased in Maxi-straws, by raising the temperature of the thawing bath. This could also be seen in the FlatPacks when comparing 35 with 50°C, but not when comparing 50 with 70°C. The different freezing rates did not affect the sperm velocity.

Table 3
The effect of freezing rate, thawing rate and boar on some motility measurements (means) made on frozen-thawed boar spermatozoa

<table>
<thead>
<tr>
<th>Variable</th>
<th>Label</th>
<th>n</th>
<th>Linear motile (%)</th>
<th>Circular motile (%)</th>
<th>V AP (μm/s)</th>
<th>LHD (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freezing rate</td>
<td>20</td>
<td>80</td>
<td>62.4ab</td>
<td>22.3 a</td>
<td>73.0 a</td>
<td>2.79 a</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>80</td>
<td>60.6a</td>
<td>22.0 a</td>
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<td>2.83 a</td>
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<td></td>
<td>80</td>
<td>80</td>
<td>64.6b</td>
<td>20.4 a</td>
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<tr>
<td></td>
<td>M70</td>
<td>48</td>
<td>61.2a</td>
<td>24.2 b</td>
<td>68.1 b</td>
<td>2.43 b</td>
</tr>
<tr>
<td></td>
<td>FP35</td>
<td>48</td>
<td>60.5a</td>
<td>22.3 b</td>
<td>76.8 e</td>
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<tr>
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<td>66.2b</td>
<td>15.9 c</td>
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<td>68.0a</td>
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<td>63.3b</td>
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</tbody>
</table>

* Values with different letters within column and variable differ significantly \( (p<0.05) \).

\( ^a \) C/min in freezing chamber.

\( ^b \) M: Maxi-straw, FP: FlatPack; 35, 50 and 70: temperature of thawing bath (°C).

\( ^c \) After adjustment for the effects included in the model.
Table 4
Relative improvement, and level of significance for some post-thaw sperm characteristics caused by freezing rate, thawing regime, and boar

<table>
<thead>
<tr>
<th>Variable</th>
<th>MI</th>
<th>Motsubj</th>
<th>Motility CASA</th>
<th>Linear motile</th>
<th>Circular Motile</th>
<th>VSL</th>
<th>VAP</th>
<th>VCL</th>
<th>LHD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freezing rate</td>
<td>3.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.8</td>
<td>9.2</td>
<td>6.6</td>
<td>8.5</td>
<td>1.2</td>
<td>1.4</td>
<td>2.5</td>
<td>4.0</td>
</tr>
<tr>
<td>ns&lt;sup&gt;b&lt;/sup&gt;</td>
<td>***</td>
<td>**</td>
<td>*</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>**</td>
<td>***</td>
</tr>
<tr>
<td>Thawing regime</td>
<td>6.3</td>
<td>33.2</td>
<td>34.3</td>
<td>9.6</td>
<td>41.8</td>
<td>23.5</td>
<td>23.3</td>
<td>31.0</td>
<td>38.5</td>
</tr>
<tr>
<td>ns</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Boar</td>
<td>14.3</td>
<td>18.6</td>
<td>13.5</td>
<td>21.2</td>
<td>32.6</td>
<td>18.8</td>
<td>16.3</td>
<td>9.1</td>
<td>1.8</td>
</tr>
<tr>
<td>***</td>
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<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>ns</td>
</tr>
</tbody>
</table>

<sup>a</sup> Relative improvement (%) = (highest group value-lowest group value)/lowest group value ×100.
<sup>b</sup> Level of significance.
<sup>c</sup> Visual motility %.

There was a significant increase in lateral head displacement when using a higher thawing temperature within package, the FlatPack yielding higher values than the Maxi-straw (Table 3).

Both freezing rate and thawing regime as well as boar showed statistically significant effects for many of the motility measures. There were considerable differences, however, when comparing the magnitude of the changes caused by the different variables. With the exception of plasma membrane integrity and linear motile spermatozoa, in which most of the variation was caused by the boar effect, thawing regime accounted for the largest proportion of the variation for all other parameters. For most variables, the lowest extreme value for different thawing regimes was for Maxi-straws thawed at 50°C, and the highest for FlatPacks thawed at 50 or 70°C. Although giving statistically significant differences, freezing rate contributed relatively little to the variation explained by the model. This is shown in Table 4, where the relative improvement, between the extreme values, in the different parameters is shown for the three variables.

4. Discussion

In a number of studies in which freezing of boar semen in Maxi-straws has been compared with various smaller and/or flatter containers, the proportion of motile spermatozoa post-thaw was lower with the Maxi-straw (Baron, 1986; Fazano, 1986; Ewert, 1988; Leps, 1988; Moura, 1988; Stampa, 1989; Bwanga et al., 1990; Wegmann, 1990; Berger and Fischerleitner, 1992; Simmet, 1993). This was also seen in the present study. Moreover, analysis of motility pattern with CASA revealed more vigorously moving spermatozoa, shown as higher velocity and lateral head displacement, in samples frozen in FlatPacks. The reason for this improvement in post-thaw sperm motility was assumed to be that the freezing and thawing was more uniform throughout the semen sample.

Spermatozoa have to traverse the critical temperature zone of −15 to −60°C during freezing and thawing, and both these events are potentially harmful. Whether the improved post-thaw quality of semen frozen in smaller straws is due to better freezing and/or thawing conditions is unclear. Fazano (1986) concluded that it was due to the faster freezing rate, as slow-freezing in 0.5 ml straws gave no advantage, compared with Maxi-straws. On the
other hand, Baron (1986) found no differences when semen was frozen at 12 or 44°C/min in the interval between −5 and −40°C.

For the Maxi-straw there have been conflicting results concerning temperature difference between different parts of the straw during freezing, with one study showing such a difference (Bwanga et al., 1990), while another claimed that the freezing conditions were almost uniform (Weitze et al., 1987). Pursel and Park (1985) found no difference in post-thaw motility and normal apical ridges (acrosomes), when comparing freezing point plateau of 1 and 5 min. It therefore seems unlikely that the differences in freezing point plateau seen in this study between Maxi-straw (45–90 s) and FlatPack (20–42 s) could be the major cause of the difference seen in post-thaw motility. In fact the duration of freezing point plateau for Maxi-straw frozen at 80°C/min (45 s) and FlatPack frozen at 20°C/min (42 s) was almost the same, and yet post-thaw motility showed substantial differences.

The most pronounced difference in the temperature courses during freezing and thawing occurred between −20 and 0°C during thawing, with a 4–5-fold increase in thawing velocity, when comparing the center of the Maxi-straw with the FlatPack. The difference between the center and periphery of the Maxi-straw, within the same temperature interval, was 3–4-fold. This agrees with Weitze et al. (1987), who noticed that the thawing rate, between −20 and −2°C, was 3.75 times slower in the periphery compared with the center of Maxi-straws. The results from the motility measurements suggest a beneficial effect of passing quickly through this temperature interval during thawing. The exact reason for this is not known, though various explanations have been proposed.

Low levels of glycerol (up to about 3%) are compatible with retained fecundity of boar spermatozoa after freezing (Watson, 1990). According to the two-factor theory of cryoinjury, the optimum freezing rate increases inversely with decreasing glycerol concentration (Mazur, 1985). The optimal freezing rate for boar spermatozoa has been reported to be 16°C/min with Maxi-straw (Pursel and Park, 1985), 30°C/min for 0.5 ml straw (Fiser and Fairfull, 1990), and 50°C/min for 0.25 ml straw (Woelders and Den Besten, 1993). We chose three different freezing rates in a rather narrow interval, 20, 50 and 80°C/min. These span probably the range of what is currently used when freezing boar semen.

The freezing rates used in this study can be considered fairly rapid, and therefore thawing should be commensurately quick in order to avoid recrystallization of intracellular ice (Watson, 1979). The presence of intracellular ice, in combination with recrystallization to larger, more stable ice crystals has been the plausible explanation of the poorer motility seen with Maxi-straws (Fiser and Fairfull, 1990). Indeed Courtens and Paquignon (1985) found microcrystals in mitochondria of about half of the spermatozoa in the frozen state. On the other hand, Gilmore et al. (1998) extrapolated plasma membrane characteristics for boar spermatozoa obtained above 0°C and found that the probability of intracellular ice formation was <5% in the presence of glycerol, when using cooling rates of 10, 100 and 1000°C/min. They argued that most cell loss predicted at high cooling rates is not associated with intracellular ice formation, but is due to excessive cell swelling upon rapid warming. It is possible, however, that suprazero water transport parameters are not to be considered valid during the type of freezing and thawing procedures assayed in the present study. This was suggested for mouse spermatozoa where differential scanning calorimetry measurements during freezing showed that intracellular ice formation could occur at cooling rates as low as 40°C/min (Devireddy et al.,
In general, rewarming in hot water has improved the motility and acrosomal integrity of ram, bull and stallion spermatozoa (see Watson (1990) for review). A rapid thawing rate is thought to be beneficial to boar spermatozoa too (Fiser et al., 1993; Westendorf et al., 1975). Also in the present study, an increased temperature of the thawing bath improved post-thaw motility within package (Maxi-straw from 50 to 70°C, and FlatPack from 35 to 50°C). However, in parallel to what Fiser et al. (1993) and Woelders and Malva (1998) found, sperm motility could not be further improved, in samples frozen in FlatPack, by increasing the temperature from 50 to 70°C. This suggests that there is a plateau in the relation between thawing rate and sperm survival.

The effect of the freezing rates used in the present study on the post-thaw motility, although statistically significant, was rather small. The range of freezing rates was probably too narrow to demonstrate any major effect on cell survival, as studies that have shown an effect of freezing rates on the post-thaw survival used a wider interval of freezing rates (Fiser and Fairfull, 1990; Woelders and Den Besten, 1993). Another explanation might be that the difference within-package (5 ml) freezing and thawing courses noted in the present study could be more pronounced than in smaller straws (0.25 or 0.50 ml), thus obscuring the effect of the freezing rates used. Nevertheless, our results suggest that instead of a fixed freezing rate, boar semen tolerates a range of freezing rates around an optimum (Watson, 1979). This has also been shown for spermatozoa from other species (Robbins et al., 1976; Rota et al., 1998).

To further complicate this, it has been reported that when boar semen was frozen, the effect of the freezing rate was pronounced in some (but not all) boars (Eriksson et al., 1997a,b; Medrano et al., 1998). This boar dependence of the freezing rate effects on sperm cryosurvival, could also explain some of the conflicting results about the importance of freezing rates, reported in the literature.

Although the proportion of motile spermatozoa is the most commonly used measure of semen quality, the correlation with fertility has often been reported as low (Saacke and White, 1972; Linford et al., 1976). With the advent of computerized assisted semen analysis (CASA), greater effort has been concentrated on the importance of different motility patterns in conjunction with fertility. Most studies in this field have been performed in the human, where lateral head displacement and sperm velocity were most closely correlated with fertility both in vitro (Holt et al., 1985; Davis et al., 1991) and in vivo (Marshburn et al., 1992; Barat et al., 1993). Recent studies on boar spermatozoa showed that these parameters of sperm motion were correlated with in vitro (Grant et al., 1994) and in vivo fertility (Holt et al., 1997) in this species too. In boars it appears that the more rapidly motile spermatozoa display greater lateral movement of the head (Holt et al., 1996), as was also seen in our study.

For hamster spermatozoa it was concluded that fertility depends on the presence of a sufficient number of spermatozoa exceeding a certain velocity threshold (Slott et al., 1997). If this is also true of boar spermatozoa, the results obtained in the present study, with a larger number of spermatozoa showing vigorous post-thaw motility when frozen in FlatPacks,
might improve the result when using frozen-thawed boar semen for artificial insemination, or allow one to reduce the number of spermatozoa used in each insemination dose, compared with that traditionally used with Maxi-straws.

We have noticed earlier that quite a number of boar spermatozoa showed circular motility, when chamber depth was 10 μm, as in this study. This was presumably due to the fact that the spermatozoa bounce against the upper and lower glass surfaces of the chamber while propelling their way through the medium. The proportion of circular motile spermatozoa is usually increased in samples with more vigorous motile spermatozoa, such that cells with less lateral head movement move more straight ahead. Somewhat surprisingly we saw in the present study that although the samples frozen in FlatPacks, and thawed at 50 and 70°C, yielded spermatozoa with higher velocity and lateral head displacement, they still contained more linear motile spermatozoa, and fewer circular motile ones. At the moment we cannot offer any explanation for this contradiction.

The higher value of percentage motile spermatozoa obtained with the CASA, compared with visual estimation is most probably due to the cell motion analyzer’s inability to correctly classify immotile spermatozoa, as has been reported earlier (Wiedermann, 1992; Togni et al., 1995).

In contrast to what was noticed regarding post-thaw motility, neither freezing nor thawing rate had any significant effect on plasma membrane integrity. This is consistent with the fact that in many studies (see Simmet (1993) for review) little or no improvement in normal acrosomal ridges (NARs) has been seen, although there was a clear improvement in motility. In fact, Simmet (1993) saw no effect on the proportion of NARs between Maxi-straws and FlatPacks in one study, but could see a significant improvement with the FlatPack when using other boars. Also for spermatozoa of other species, motility has been shown to deteriorate before any change was seen in plasma membrane integrity (McLaughlin et al., 1992; Liu and Foote, 1998; Polcz et al., 1998) when cryopreserved or exposed to anisotonic conditions.

We also saw a significant boar effect on post-thaw membrane integrity, indicating that factors within boar are more decisive for this parameter than were the freezing and/or thawing rates used here. Of course % NARs and % membrane intact spermatozoa are not directly mutually equivalent, but both are measures of membrane status and, indirectly, of sperm viability. It seems from the present study that although plasma membrane integrity measured with fluorescent probes can be used to monitor the degree of cell death during freezing and thawing, it says little about the more subtle changes that spermatozoa exhibit as a result of such treatment. As suggested by Holt and Medrano (1997) there is probably a wide variation in spermatozoal fertilizing capacity within the so called viable population.

The higher values noted for plasma membrane integrity compared with subjective motility, with 10 and 20% points for FlatPack and Maxi-straw, respectively, deserve some comment. This is often seen when using several methods for assessing sperm survival post-thaw. It is plausible that the number of fertile spermatozoa is considerably lower than either motile spermatozoa or those with an intact plasma membrane. In the present study we used a few methods to measure the quality of the spermatozoa post-thaw. However, spermatozoa need a whole array of functions intact to fulfill their duty when present in the female genital tract (Amann and Hammerstedt, 1993).
5. Conclusion

The FlatPack gave better post-thaw motility than the Maxi-straw, probably by allowing a quicker rate of thaw. Plasma membrane integrity was not affected by either freezing or thawing rate. Along with the freezing and thawing rates, extenders, packages and boars used in the present study, thawing rate had the greatest influence on post-thaw sperm survival followed by boar and freezing rate.

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References


