COMPARATIVE VIABILITY OF BOVINE SPERM FROZEN ON A CRYOMICROSCOPE OR IN STRAWS

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ABSTRACT

The accuracy and repeatability of freezing rates and effects of evaporation were examined using a new cryomicroscope system to establish its usefulness in assessing the development of cryopreservation protocols for bovine semen. Post-thaw sperm plasma membrane integrity, as assessed by using combinations of fluorescent stains and flow cytometry, was used in evaluating protocols for freezing spermatozoa on the cryomicroscope. Semen was diluted in Test-yolk (20%) extender containing 7% glycerol and frozen in 0.5-ml straws, 0.25-ml straws (over liquid nitrogen for 8 min) or in a quartz crucible using a Linkam BCS 196 cryomicroscope. Thawed samples were diluted with Hepes buffered medium containing 0.1% bovine serum albumin (BSA) and stained with either carboxymethylfluorescein diacetate (CMFDA) or SYBR-14 each in combination with propidium iodide (PI). Flow cytometry analysis of the samples revealed 2 major populations: 1) spermatozoa with intense green fluorescence (stained with CMFDA or SYBR-14), which were classified as plasma membrane-intact and 2) spermatozoa with intense red fluorescence, (stained with PI), which were classified as plasma membrane-damaged. Samples frozen using the cryomicroscope contained 29 and 26% plasma membrane-intact (PMI) sperm cells, as assessed by CMFDA and SYBR-14, respectively. Cryopreservation of spermatozoa in 0.5-ml straws resulted in 22 and 20% plasma membrane-intact sperm cells, while spermatozoa frozen in 0.25-ml straws resulted in 34 and 31% PMI sperm cells for CMFDA and SYBR-14, respectively. No significant difference was observed (P>0.05) for PMI spermatozoa stained with either CMFDA or SYBR-14. In addition, the ability to recover spermatozoa after freezing on the cryomicroscope establishes the Linkam BCS 196 as a useful tool for the study of sperm cell cryopreservation.

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Key words: cryomicroscopy, flow cytometry, fluorescent stains

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INTRODUCTION

Artificial insemination (AI) of cattle with frozen-thawed spermatozoa represents the most effective use of gamete cryopreservation technology in domestic animals. Even though more than 80% of all dairy cattle are artificially inseminated using frozen-thawed semen, 10 to 15 times more spermatozoa are required per insemination than when fresh semen is used (19) for insemination. Damage to bovine spermatozoa during the freezing-thawing process has been well documented (17, 8), suggesting that loss of plasma membrane integrity is one of the primary indicators of the loss of sperm function. This has also been demonstrated in other domestic species (16, 11).

Reducing damage during freezing-thawing procedures, particularly to the plasma and acrosomal membrane, would decrease the number of spermatozoa needed per insemination, thus increasing reproductive efficiency when cryopreserved spermatozoa are used. Methods for evaluating sperm viability and functional integrity before, during and after cryopreservation would be advantageous to the development of new freezing-thawing protocols. Sperm motility and acrosomal integrity measured before and after freezing and thawing are useful indicators of sperm damage (16, 4, 6).

Combining cryomicroscopy with flow cytometry analyses of fluorescently stained sperm cells to determine membrane integrity offers a more specific assessment of sperm viability in spermatozoa subjected to freezing and thawing during the standard cryopreservation procedures. The cryomicroscope provides an opportunity for evaluating spermatozoa during freezing and thawing. Assessment, however, is often limited by the small volumes (1.4 μl) used for accurate cooling on the cryostage that are difficult to recover after freezing and thawing. A new cryomicroscope system, the Linkam BCS 196, was developed which is capable of freezing volumes of up to 100 μl through use of a quartz crucible, thereby allowing post-thaw evaluation of samples. Fluorescent staining techniques in combination with flow cytometry offer the ability to evaluate membrane integrity of thousands of individual sperm cells within a few minutes. Monitoring the viability of sperm using flow cytometry and staining with carboxyfluorescein diacetate (CMFDA) has proven successful (7). More recently, fluorescent staining and flow cytometry analysis of spermatozoa has been demonstrated for several species using the new nucleic acid stain, SYBR-14 (6).

The objectives of this study were to validate the Linkam BCS 196 cryomicroscope for the development of cryopreservation protocols for bull spermatozoa and to combine this technology with assessment of sperm membrane integrity using fluorescent staining and flow cytometry analysis. Three areas of sperm cryopreservation were investigated: 1) the accuracy of the cooling rates of the cryomicroscope system, 2) the effects of condensation or evaporation on freezing large volumes (100 μl) in the quartz crucible, and 3) the repeatability of freezing aliquots from the same ejaculate. A fourth objective was to compare viability of spermatozoa frozen in straws with those frozen on the cryomicroscope. Finally, the percentages of PM1 sperm cells as determined by supravital stains CMFDA and SYBR-14, were also compared after freezing bull spermatozoa in straws and on the Linkam BCS 196 cryomicroscope.
MATERIALS AND METHODS

Semen Preparation

Ejaculates from three bulls were collected three times over nine days. The semen was diluted to $60 \times 10^6$ sperm/ml in modified Test-yolk (20%) extender containing 0.1% Equex (12). Samples in 5 ml polystyrene tubes were transferred to a cold room (4°C) in a beaker containing 20 ml of water at room temperature (24°C) for two hours until the temperature reached 4°C. An equal volume of Test-yolk extender containing 14% glycerol was added to the diluted semen to give a final concentration of $30 \times 10^6$ sperm/ml, and 7% glycerol.

Cryomicroscope

The Linkam BCS 196 consists of a modified microscope stage and a cryostage (Linkam Scientific, Surrey, UK), adapted for a Zeiss Axioskop microscope (Carl Zeiss, Hanover, MD). The cryostage consists of a silver block, 22 mm in diameter. Liquid nitrogen (LN$_2$) is introduced directly to produce accurate and rapid cooling rates. A small tab of silver mounted to the cooling inlet at the edge of the stage provides a seeding point to initiate ice crystal formation. The heating element is cast into the cryostage to form an integral heater and sensor assembly. A platinum resistor mounted near the surface of the cryostage provides a stable and accurate temperature signal. Sample carriers have been designed to hold either a 16 mm cover slip for freezing thin layers or a 16 mm quartz crucible for freezing larger volumes. The Linkam BCS 196 also includes a programmable unit which allows the programming of specific cooling and warming rates.

Staining Procedure

Carboxyfluorescein diacetate was prepared by dissolving 1 mg in 1 ml of anhydrous dimethyl sulfoxide (DMSO; Aldrich Chemical Co., Milwaukee, WI; 7). Both SYBR-14 and PI were obtained from Molecular Probes, Inc. Eugene, OR. They were prepared by dissolving 1 mg of SYBR-14 in 1 mg of DMSO and 2 mg of PI in 1 ml of Dulbecco's PBS (6). All stains were stored at 4°C away from light. Working dilutions of stock were 0.57 μl/ml for CMFDA and SYBR-14, and 4 μl/ml for PI. Stain was delivered to each sample using a P-2 and P-10 Pipetman (Rainin Instrument Co., Emeryville, CA) and Fluoropel pipette tips (Fischer Brand READI-TIP P-2 low retention cat. # 21-276A, Fischer Scientific, Santa Clara, CA) to ensure the accurate delivery of the small volumes used in this study.

Flow Cytometry Analyses

Samples were run at a rate of 10 μl/min on an Epics Profile II equipped with a 15-mw argon laser at 488 nm (Coulter Corporation, Inc., Miami, FL). Log fluorescence was collected through LFL1 and LFL3 635-nm band pass filter channels, and 10,000 sperm cells were analyzed using a dotplot two-channel cytogram on the Epics Profile II histogram analysis program. Electronic compensation was used to minimize spillover of green fluorescence into the 635-nm
red channel (LFL3). Debris and other particles, (i.e., droplets) were eliminated by gating only those cells having the light scatter properties of spermatozoa. The resultant sperm populations were quantified for fluorescent intensity by gating on populations falling within specific channels and were assigned percentages according to the relative number of cells and then analyzed as previously described (6).

Experiment 1. Validation of the Cryomicroscope

**Cooling rate consistency.** To investigate the consistency and accuracy of the cryostage cooling, 4 different rates were examined. The temperatures recorded from the readout of the cryostage and from an independent thermomister probe were compared. A fine wire (0.41 mm) thermistor probe (Physitemp Instruments, Inc., Clifton, NJ) was imbedded in heat sink compound on the surface of the silver cryostage and attached by covering with adhesive tape. Four different programmable rates were examined: 1) room temperature (~24°C) to -70°C at a rate of 20°C/min; 2) room temperature to -70°C at a rate of 50°C/min; 3) room temperature to -140°C at a rate of 80°C/min; and 4) room temperature to -140°C at a rate of 100°C/min. Recordings were taken at 15-sec intervals from both the cryomicroscope readout and the thermistor probe until the target temperature had been reached and held for 1 min, or until the temperature readings remained unchanged for 1 min. Each cooling rate program was replicated 3 times.

**Osmolarity of sodium chloride solution after freezing and thawing in a quartz crucible.** To investigate the effects of possible evaporation of 2 different volumes during freezing and thawing in the quartz crucible, the osmolarity of samples was measured before and after freezing using a vapor pressure osmometer (Westcor, Inc., Logan, Utah). Standard sodium chloride solutions prepared as 290-mOsm calibration controls for osmometer readings were used. Volumes of 50 and 100 μl were placed in a quartz crucible on the cryomicroscope at 4°C and held for 1 min before cooling at a rate of 10°C/min to -70°C. The samples were thawed at a rate of 30°C/min to 37°C. Osmolarity of the sample was measured at 4°C before freezing and at 37°C after thawing in 18 aliquots for each volume tested.

**Repeatability of freezing aliquots from the same ejaculate.** Extended, glycerolated semen was maintained at 4°C before being transferred to the cryomicroscope. The quartz crucible was placed onto the cryostage at room temperature and was cooled to 4°C before 100 μl of diluted semen was added to the crucible. Initial cooling was at 4°C/min to -8°C. At this temperature the samples were seeded by moving the crucible over to the silver seeding tab at the edge of the silver block until the initial formation of ice crystals was observed. Seeded samples were further cooled at a rate of 40°C/min to -70°C and held for 1 min before thawing at 100°C/min to 37°C. Four aliquots were frozen from each of the 9 ejaculates, and the percentages of PM1 spermatozoa were determined by staining with SYBR-14 and PI and flow cytometry analysis as described above.

Experiment 2. Comparison and Evaluation of Cryopreserved Sperm Using Fluorescent Staining

**Experimental design.** Ejaculates from 3 bulls were collected 3 times over 9 d, in random
order. Treatments were allocated in a 3 x 2 factorial design. Extended, cooled, glycerolated semen was frozen in either 0.25- or 0.5-ml straws, or on the cryomicroscope. Thawed samples were stained with 2 combinations of fluorescent viability stains (CMFDA and PI or SYBR-14 and PI) and analyzed by flow cytometry. For each of the 9 d, the percentage of PM1 sperm cells was quantified in duplicate for each freezing method by stain combination.

**Cryomicroscope protocol.** The extended, cooled, glycerolated semen was maintained at 4°C. The cryostage was initially flushed with LN2 to precool the tubing and clear residual water. The quartz crucible was then placed on the stage, in the holder. A controlled freezing rate designed for freezing straws in a programmable cell freezer was chosen for the cryomicroscope (3). After cooling to 4°C, 100-μl samples were transferred to the crucible and cooled at a rate of 4°C/min to -8°C. At this temperature samples were seeded by moving the crucible over to the seeding tab at the edge of the stage. The samples were then cooled at 40°C/min (3) to -70°C and held at this temperature for 1 min before thawing at a rate of 100°C/min to 37°C. Thawed spermatozoa were added to 300 μl of Hepes buffered medium (130 mM NaCl, 4 mM KCl, 14 mM Fructose, 1 mM CaCl2, 0.05 mM MgCl2 and 1 mM Hepes) containing 0.1% bovine serum albumin (BSA;9) at 37°C and stained with a combination of CMFDA and PI or SYBR-14 and PI, and then incubated for 15 min at 37°C. Sperm viability was analyzed by flow cytometry as described in Experiment 1.

**Straw freezing protocol.** French straws (0.25 and 0.5 ml) were filled with extended, cooled, glycerolated semen at 4°C and sealed with polyvinyl alcohol powder. Freezing of straws was carried out in the vapor phase, ~3 cm above the level of LN2, for 8 min before plunging into LN2 for a minimum of 24 h. The straws were thawed by immersing them in water at 37°C for 15 sec (0.25-ml straws) or 30 sec (0.5-ml straws). The straws were wiped dry and the contents released into 0.75 and 1.5 ml of Hepes buffered medium containing 0.1% BSA at 37°C for 0.25 and 0.5-ml straws, respectively. Thawed, diluted spermatozoa were then stained with a combination of CMFDA and PI or SYBR-14 and PI, for 15 min at 37°C before analysis by flow cytometry as described above.

Statistical analysis

Student's t-test was performed using SAS least square means (18) to determine significant differences between the osmolarity of the samples before and after freezing. Data collected to evaluate the repeatability of freezing aliquots from the same sample were fitted to a random effects model in order to investigate the variance components for bull, day and subsamples using the SAS MIXED procedure (18). Variance components were compared to zero with the F-test using the SAS GLM procedure (18). The standard deviation of the percentage of PM1 sperm cells was determined in this study. The percentage of PM1 sperm cells for the comparison of CMFDA and SYBR-14 staining procedures was fitted to a linear mixed model using the SAS procedure MIXED (18), where bull and day were considered random effects. Variance homogeneity was confirmed by examining the residuals. The main effects and interactions for the 3 x 2 factorial (freezing method by stain) were also tested. In standard ANOVA for balanced designs, the standard errors of the treatment means are equal because the variance of the observations is assumed to be equal.
RESULTS

Experiment 1. Validation of the Cryomicroscope

Cooling rate consistency. All cooling rates obtained from the cryomicroscope were accurate to within 1°C when compared with those of the independent thermister probe. All 4 rates were highly consistent when repeated. Target temperatures were reached without decreasing the freezing rate.

Osmolarity of a sodium chloride solution after freezing and thawing in a quartz crucible. A decrease of 5 and 10 mOsm was observed in the 50- and 100-μl samples, respectively, after freezing at a rate of 10°C/min to -70°C and thawing at a rate of 30°C/min (P=0.01; Table 1).

Table 1. Mean osmolarity of a standard sodium chloride solution (290 mOsm) at 4°C before freezing and at 37°C after freezing on a Linkam BCS 196 cryomicroscope

<table>
<thead>
<tr>
<th>Volume</th>
<th>Replicates</th>
<th>mOsm ± SEM</th>
<th>4°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 μl</td>
<td>n=18</td>
<td>288 ± 0.6a</td>
<td>283 ± 1.8b</td>
<td></td>
</tr>
<tr>
<td>100 μl</td>
<td>n=18</td>
<td>289 ± 0.5a</td>
<td>279 ± 1.8b</td>
<td></td>
</tr>
</tbody>
</table>

Rows with different subscripts are significantly different (P<0.01).

Repeatability of freezing aliquots from the same ejaculate. The difference in the number of PM1 spermatozoa between ejaculates was highly significant on different days (P<0.001) but not for individual bulls (P=0.32). The number of PM1 sperm cells assessed by flow cytometry, was not significantly different (P>0.05).

Experiment 2. Comparison of Viability Staining Using CMFDA and SYBR-14

The interaction between freezing method and stain was not significant at the P=0.05 level, (i.e., there was no difference in the percentage of PM1 spermatozoa after staining with CMFDA and SYBR-14 for any of the freezing methods; Table 2). As there were no interactions between stain and freezing method, the main effect means were examined to assess the effect of freezing method on PM1. Higher percentages of PM1 spermatozoa were obtained after freezing in 0.25-ml straws than frozen on the cryomicroscope or in 0.5-ml straws (P<0.01; Table 2).
Freezing on the cryomicroscope resulted in a higher percentage of sperm cells with intact membranes than freezing in 0.5-ml straws (P=0.01; Table 2).

Table 2. Percentage means and main effect means of PM1 bull spermatozoa after staining with CMFDA or SYBR-14 following freezing in straws or on a Linkam BCS 196 cryomicroscope

<table>
<thead>
<tr>
<th>Freezing method</th>
<th>Stain</th>
<th>Main effect of freezing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CMFDA</td>
<td></td>
</tr>
<tr>
<td>0.25 ml-straws</td>
<td>34</td>
<td>32^a</td>
</tr>
<tr>
<td>Cryomicroscope</td>
<td>28</td>
<td>28^b</td>
</tr>
<tr>
<td>0.5-ml straws</td>
<td>22</td>
<td>21^c</td>
</tr>
</tbody>
</table>

Main effect of staining 26d 28d

--- SEM=2.0 ---

^a,b,c Column main effect means with no common superscript are significantly different (P<0.01).
^d Row main effect means followed by the same letter are not significantly different (P=0.08).

DISCUSSION

Bull spermatozoa were successfully recovered for analyses by flow cytometry after freezing on the Linkam BCS 196 cryomicroscope. Accurate and consistent cooling rates, within the range of rates normally used for freezing bull semen (1, 8, 3), were obtained using the Linkam cryomicroscope. In addition, repeated freezing and thawing of multiple samples from the same ejaculate resulted in little variation between numbers of PM1 sperm cells. The cooling rate during cryopreservation has been identified as a crucial factor in determining human sperm survival (10). Water and cryoprotectant movement across the membrane is dependent on the cooling rate, and this, in turn, affects the survival of spermatozoa. Erratic cooling rates could result in fluctuations in the movement of water and cryoprotectant across the plasma membrane and result in cell damage. The silver cryostage of the Linkam BCS 196 has high heat conductivity, which allows the cryostage to be cooled accurately by decreasing temperature fluctuations of the cryostage.
When freezing volumes of 50 and 100 µl in the quartz crucible, an increase in osmolarity is expected due to the expected evaporation of the sample. However, the converse was observed, and osmolarity decreased. Closer inspection revealed the presence of small ice crystals that had formed around the perimeter of the crucible. These crystals probably resulted from moisture in the air condensing on the crucible during cooling. During thawing, these crystals could have diluted the sample, resulting in decreased osmolarity. Thus a slower rate of cooling was used for the osmolarity experiments than for the freezing experiments to maximize any effect of evaporation. However, the slight decrease in osmolarity, which was observed after thawing, would have had only a minimal impact on sperm survival after freezing in the quartz crucible. The formation of ice crystals is a commonly encountered problem with cryomicroscope experiments (14).

Recent studies have shown that SYBR-14 can be used to effectively evaluate PMI in bull spermatozoa (6) as well as in the spermatozoa from several other mammals (5). In our present study, SYBR-14 was effective in assessing the integrity of plasma membranes of intact bull spermatozoa compared with that of CMFDA. Moreover, SYBR-14 has several advantages over CMFDA. The SYBR-14/PI combination becomes comparatively stable after 15 min of incubation (6), thus staining times and temperatures are less critical than for the esterase-dependent stain, CMFDA. When the plasma membrane integrity was breached, SYBR-14 staining was replaced by PI, providing more rapid response than CMFDA, which may require longer to be released from the entire sperm cell. The rapid response of SYBR-14 change to PI staining may not be noticeable with gross membrane damage, but may be useful in detecting minor membrane damage. In this study, CMFDA and SYBR-14 stained cells similarly. These 2 precise viability stains are advantageous in the assessment of frozen/thawed spermatozoa because they provide quantifiable estimates of PMI.

A large variance in the percentage of frozen/thawed PMI spermatozoa was seen between the days on which the samples were collected. Although a difference in the number of PMI spermatozoa after freezing and thawing might be expected to be due to variation in individual animals, no bull effect was observed in this study. Spermatozoa from all 3 bulls showed similar viability assessments following cryopreservation and thawing.

To determine how freezing spermatozoa on the Linkam BCS 196 cryomicroscope compares with that of conventional straw freezing methods, spermatozoa were frozen using both methods. The cooling rate chosen for the cryomicroscope may not be optimal for freezing spermatozoa on a cryostage, since the PMI was lower on the cryomicroscope compared with that of 0.25-ml straws in the present study. Caution must be exercised when drawing direct comparisons between the freezing methods used in this study, since it is important to note the differences in the types of freezing that occurs in straws and on the cryomicroscopes (9). The Linkam BCS 196 allowed the sample to be held at a specific temperature for seeding of the sample. This minimizes the incidence of supercooling which can be detrimental to living cells (13). In contrast with freezing in the straws, the point of ice crystal formation is held steady on the cryomicroscope and, therefore, the entire sample is frozen before cooling is continued. In straws, the surrounding temperature continues to drop after the sample has started to freeze. This may result in a portion of the sample freezing at a much lower temperature than that part of the
straw where ice crystal formation was initiated. The varying percentages of membrane-intact spermatozoa following freezing in 0.25- and 0.5-ml straws may be attributed to the fact that a larger surface area to volume ratio is obtained with the smaller diameter straw, providing faster cooling of the sample. Osmotic stresses and cell dehydration (8) may be lower in 0.25-ml straws than in 0.5-ml straws. On the cryomicroscope, a larger surface area is in direct contact with the cooling block, thus a larger relative volume is uniformly cooled. In spite of these differences, freezing on the cryomicroscope provides an effective way for examining different protocols in the development of enhanced semen cryopreservation methods that might then be transferred to containers such as straws or vials (15).

In the present study, bull spermatozoa were successfully recovered after freezing on a cryomicroscope and then evaluated using fluorescent stains and flow cytometry analysis. In addition, repeated freezing and thawing of multiple samples from the same ejaculate resulted in very little variation between sperm aliquots. Predictive measures of sperm freezing techniques are proving difficult to obtain. Studies on sperm membrane permeability co-efficients failed to predict optimal cooling rates (2); therefore, the development of sperm freezing protocols is still empirical. In developing experimental protocols (i.e., comparisons of cryoprotectants or freezing/thawing rates), the ability to accurately and consistently freeze samples would limit error due to procedural variability such as that of fluctuations in the cooling rate. The use of cryomicroscopy together with flow cytometry allows for accurate and consistent freezing and thawing rates, with rapid sperm plasma membrane assessments conducted at critical intervals. The combination of these technologies allows for a more precise evaluation of factors influencing sperm freezing, and should prove to be an effective tool in the development of sperm cryopreservation protocols.

REFERENCES


