Large Batch Freezing of Bull Semen: Effect of Time of Freezing and Fructose on Fertility

R. H. Foote* and M. T. Kaproth†
*Department of Animal Science
Cornell University, Ithaca, NY 14853
†Genex Cooperative, Inc., Shawano, WI 54166

ABSTRACT

Large-scale batch freezing of bull semen should be done in a processing schedule that yields the highest fertility and when it can be fitted efficiently into the work schedule. Conflicting reports have appeared on survival and fertility of bull sperm frozen within a few hours of semen collection or on the next day. To study this problem, a factorially arranged experiment with semen from 10 bulls was conducted, comparing whole milk-glycerol semen extender with and without fructose, and semen frozen in 0.5-ml straws after 4 versus 18 h of equilibration at 5°C. Both fructose and 18 h of equilibration resulted in a small but significant improvement in freeze-thaw survival of sperm. A field trial followed with replicated semen collections from nine bulls processed in a whole milk-glycerol control extender frozen after 4 h of equilibration versus the addition of 1.25% (wt/vol) fructose to whole milk glycerol divided to freeze sperm after 4 and 28 h of equilibration. Semen from these bulls was used to inseminate 14,775 first-service cows. The 56-d nonreturn rates obtained for these three treatments were 74.7, 74.3, and 73.9%, respectively. As there was no difference in fertility, it would appear that programs to freeze sperm in whole milk extenders the same day of collection or the day after semen collection should yield equivalent results.

(Key words: frozen bull sperm, equilibration time)

Abbreviation key: WM = whole milk, WMF = whole milk-fructose.

INTRODUCTION

When frozen bull semen was first being tested commercially, many factors were considered, such as extenders, semen cooling rate, glycerol concentration, and time and method of addition, packaging for freezing, and freezing conditions (Maule, 1962). In an extensive review of the literature Pickett and Berndtson (1978) concluded that slow cooling and an equilibration period for sperm at 5°C was necessary to obtain maximal fertility. Originally, the equilibration period was thought to be important for glycerol equilibration, but glycerol penetrates bull sperm rapidly, and no extensive, glycerol equilibration time is required (Berndtson and Foote, 1969; Foote, 1970; Pickett and Berndtson, 1978). Consequently, many AI organization established a routine of processing and packaging large numbers of units (ampules and later straws) for freezing the same day.

However, several studies (see Pickett and Berndtson, 1978) indicated that holding semen for 18 h overnight before freezing resulted in increases of 4 to 8 percentage units in nonreturn rates. Holding semen overnight before freezing was particularly convenient for semen custom-collected in the field. Several of the studies with different types of extenders used in delayed freezing protocols included fructose. Martin and Emmens (1961) compared holding periods of 30 min versus 18 h for egg yolk-citrate extender with and without fructose. There was no difference among most treatments, but a significant improvement in fertility was obtained by including 1.25% fructose in the extender and holding the semen for 18 h before freezing. Martig and Almquist (1966) used glass ampules and compared semen frozen in control whole milk extender without fructose held for 2 h before freezing versus whole milk containing 1.25% fructose and held for 18 h before freezing. These authors recommended that semen be held overnight before freezing, because higher fertility was obtained after holding semen for 18 h. However, the addition of fructose and the longer holding time were confounded in the experimental design, so it was not possible to isolate the two effects.

With mergers and centralization of AI facilities, larger groups of bulls are collected each day, making it more difficult to process all semen collected within a normal working day. No studies were found comparing bull sperm processed in whole milk with and without fructose when held overnight before freezing versus 4 h of equilibration before freezing.
MATERIALS AND METHODS

Bulls and Experimental Design

Semen was obtained from Holstein bulls in regular AI service housed at Genex Cooperative, Inc. (Headquarters at Shawano, WI) at Ithaca, NY. Two experiments were conducted. Experiment 1 was a sperm motility study arranged as a factorial with 10 bulls × 2 extenders (whole milk-glycerol, WM, or whole milk-glycerol fructose, WMF), 2 equilibration times before freezing sperm (4 vs. 18 h), 2 straw positions (straws held vertically versus horizontally until placed horizontally in racks for freezing), 2 thaw temperatures (30 vs. 37°C). Each semen collection from each bull was divided to provide five straws containing 20 × 10⁶ total sperm per straw for each treatment before thawing. Two straws from each treatment were thawed in a waterbath at 30 and at 37°C. The coded straws were prepared by one individual for independent microscopic evaluation of the percentage of motile sperm by two other trained observers. The fresh wet smears of sperm were placed on a slide on a warming stage at 37°C and projected onto a TV monitor for viewing. Multiple fields were examined subjectively, and the average percentage of motile sperm estimated to the nearest five percentage units.

Experiment 2 was a fertility trial. Because of the need to produce a substantial number of straws per treatment per bull that would be used to inseminate first-service cows, all ejaculates obtained from a given bull on that day were pooled and were assigned to one treatment. The treatments originally planned were two extenders (WM and WMF), and two equilibration times (4 h and frozen the next day at 28 h with the new batch of semen). However, to obtain a large number of services rapidly on each bull-treatment it was necessary to limit the test to three treatments, the control WM frozen after 4 h and the WMF frozen after 4 and 28 h. Based upon the results of experiment 1, all straws were held in the conventional horizontal position. Twelve bulls were assigned to the trial, with six bulls collected per day so that semen from two bulls was processed in each of the three treatments per day. The remaining six bulls had semen processed similarly on other days. The sequence of treatments was altered so that semen from all bulls was processed in all treatments on three successive collection dates. All bulls completed two replicates of the design.

All data were subjected to a general linear model of analysis of variance (SAS, 1985). Bulls were random and other components of the model were fixed. Because the popularity of three bulls decreased as sire proofs were used extensively on each treatment, and are included in the analysis of fertility data. Differences were tested by Tukey's HSD test, and when P < 0.05 they were considered to be significant. Also, correlations of fertility results among the three treatments (T1:WM, 4 h; T2:WMF, 4 h; and T3:WMF, 28 h) were calculated across bulls in experiment 2.

Extender and General Semen Processing

The control semen extender was homogenized whole milk (WM) heated to 95°C for 10 min, cooled, and filtered. Low levels of antibiotics were added, followed by the addition of glycerol to one fraction. Two fractions of control WM were prepared daily: fraction A was heated WM and high levels of antibiotics (Lorton et al., 1988) without glycerol, and fraction B contained 14% glycerol (vol/vol). Fraction A of the experimental WM extender was the same as the control with no glycerol or fructose. Fraction B contained 2.0% fructose (wt/vol) and 14% glycerol (vol/vol) in experiment 1 and 2.5% fructose in experiment 2.

At least two ejaculates of semen were obtained from each bull on any single morning. Each ejaculate of semen was placed in a water bath at 35°C also containing a flask of WM, fraction A. Semen was subsampled as soon as collected to measure semen quality. The sperm concentration was estimated by optical density, using a calibrated spectrophotometer. The percentage of motile sperm was estimated at 37°C with the aid of a TV monitor connected to a microscope.

The remainder of the semen was extended 1:4 (vol/vol) with fraction A of the WM extender, placed in a water jacket at 35°C, and cooled for 3 h to 5°C. After cooling, the two or more partially extended ejaculates of semen from each bull were combined and further extended with cold WM, fraction A, to one-half the final volume. Then the fraction A was appropriately divided to accommodate the four treatments in experiment 1 and three treatments in experiment 2. An equal volume of WM containing 14% glycerol (vol/vol) or glycerol plus fructose was added.

The 0.5-ml straws were processed to contain 20 × 10⁶ total sperm. The straws were sealed and placed horizontally on racks, and control semen was frozen within 4 h after glycerol addition. The cooling rate was approximately −15°C/min from +5°C to −100°C, and then straws were transferred to liquid nitrogen. Straws held for longer periods of time, as described previously, were frozen similarly. All straws were coded to identify the treatments, but the specific treatment that was represented by each code was unknown to evaluators in experiment 1 and to inseminators in experiment 2.

The straws from each collection and the treatment of semen in experiment 2 were randomly distributed.
over a large group of technicians for AI soon after processing. Thus, possible sources of variation in the field were distributed as equally as possible across treatments. Inseminators thawed semen in a coverall pocket (Bean, 1972), which under laboratory controlled conditions, thaws the semen at approximately the same rate as in a 30°C water bath (Foote and Arriola, 1987). A subset of straws was retained for quality control and for additional studies on the percentage of motile sperm after freezing and thawing.

RESULTS

Experiment 1

The percentages of motile sperm for the main variables following freezing and thawing are summarized in Table 1. Overall WMF and 18 h of equilibration resulted in higher sperm motility (P < 0.01). In addition, there was a significant interaction of extender with thawing temperature (P < 0.05). The percentages of motile sperm for WM thawed at 30 and 37°C and WMF thawed at 30 and 37°C, respectively, were 42.9, 45.7, 46.3, and 45.6. This indicates that thawing at 37°C was slightly better for WM and 30°C for WMF.

Experiment 2

Initial quality of semen assigned to each of the three treatments was similar (P > 0.05). The weighted results, based on 56-d nonreturns to first service, are in Table 2. Treatments did not differ (P = 0.90). As usual, bulls differed (P < 0.01) in nonreturn rates, ranging from 68.9 to 79.1%. As treatments did not differ, and there was no bull × treatment interaction, it was of interest to correlate the fertility results among the three treatments (T1:WM, 4 h; T2:WMF, 4 h; and T3:WMF, 28 h) across bulls. The correlations between T1:T2, T1:T3, and T2:T3 were 0.78, 0.91, and 0.68, respectively. The correlations were substantial, but reveal incomplete repeatability. This is primarily due to the binomial variation and random sources of field variation (Oltenacu et al., 1980), as in the present trial with an average of 192 first inseminations per subclass two standard deviations would be ±8.5%.

DISCUSSION

The 4 h of equilibration time chosen for experiment 1 represented a convenient time to have large volumes of extended and cooled semen aspirated into coded straws, sealed and prepared for freezing. In addition, numerous studies on glycerolating and holding semen before freezing suggested that 4 h was adequate for chilled sperm to become equilibrated for freezing (Pickett and Berndtson, 1978). Graham et al. (1957) had reported 75-d nonreturn rates of 63.4, 65.2, and 67.8%, respectively, for sperm held in whole milk-glycerol for 4, 8, and 12 h before freezing. While this report suggested that an equilibration time longer than 4 h was beneficial, 12 h holding did not result in a convenient time to freeze the semen.

Because 18 h of equilibration had been used successfully by several research groups (Pickett and Berndtson, 1978), and was a convenient overnight holding time, this time was included in experiment 1. The results (Table 1) indicated that this time interval gave good freeze-thaw survival of bull sperm, consistent with published reports. No reports were found in the literature comparing horizontal with vertical storage. The vertical storage for 18 h took less space as the straws were stored vertically in cans, and the horizontal straws were held in the racks used for freezing. The freeze-thaw survival was similar in both groups. Visual checks of straws stored vertically showed that sperm had settled considerably toward one end, while those stored horizontally had sperm layered on the lower side. With the sperm extended to 40 × 10⁶/ml (20 × 10⁶ per straw) the sperm packing that occurred had no effect on survival. There was no substantial difference in results

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**Table 1.** The percentage of motile sperm associated with the main variables in experiment 1.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Motile sperm (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extenders</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WM⁴</td>
<td>44.2</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>WMF⁵</td>
<td>46.0</td>
<td></td>
</tr>
<tr>
<td>Equilibration time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 h</td>
<td>44.1</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>18 h</td>
<td>46.2</td>
<td></td>
</tr>
<tr>
<td>Straw position</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horizontal</td>
<td>45.4</td>
<td>= 0.41</td>
</tr>
<tr>
<td>Vertical</td>
<td>44.9</td>
<td></td>
</tr>
<tr>
<td>Thaw temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30°C</td>
<td>44.7</td>
<td>= 0.08</td>
</tr>
<tr>
<td>37°C</td>
<td>45.7</td>
<td></td>
</tr>
</tbody>
</table>

¹Bulls differed (range of 31.8 - 47.4%, P < 0.01). Duplicate straws did not differ (P = 0.14).
²WM = Whole milk and WMF = whole milk-fructose extenders.

**Table 2.** Fertility results in experiment 2.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of 1st services (%)¹</th>
</tr>
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<tbody>
<tr>
<td>WMF, 4 h equilibration</td>
<td>4552 74.7</td>
</tr>
<tr>
<td>WMF², 4 h equilibration</td>
<td>4820 74.3</td>
</tr>
<tr>
<td>WMF, 28 h equilibration</td>
<td>5403 73.9</td>
</tr>
</tbody>
</table>

¹Fertility as not different (P = 0.90).
²WM = Whole milk and WMF = whole milk-fructose extenders.
between thawing the straws in 30 versus 37°C water. Therefore, it is likely that the fertility results obtained with the pocket thaw procedure would be applicable also to AI organizations thawing semen in water at about 37°C.

The fertility trial (Table 2) indicated that there was no advantage or disadvantage of adding fructose and holding semen for 4 vs. 28 h. This is in contrast to the results of Martig and Almquist (1966) who froze sperm in WMF in ampules with \(3 \times 10^6\) motile sperm. They obtained a 4.8-percentage-point advantage with sperm equilibrated for 18 h. Martin and Emmens (1961) also reported an advantage of holding sperm for 18 h in egg yolk-citrate when it contained 1.25% fructose. Their studies were performed with \(15 \times 10^6\) sperm frozen in ampules. In an extensive review of the literature Pickett and Berndtson (1978) discussed the fact that optimal holding time before freezing may depend upon the quality of semen, and different extenders and processing procedures.

Salisbury (1968) analyzed extensive fertility data with liquid semen and found a significant increase in fertility of semen used the day after collection versus the day of collection. He speculated that some of the weaker sperm might no longer be competitive after 1 d of storage at 5°C.

These are the first studies comparing sperm held for varying periods in WM or WMF and frozen in 0.5-ml straws. The dose of sperm used for insemination was intermediate between the doses used by Martin and Emmens (1961) and Martig and Almquist (1966). If there is an effect on fertility of holding sperm for 18 to 28 h in WMF, compared with shorter holding times before freezing, low numbers of sperm inseminated or perhaps sperm from subfertile bulls would likely be necessary to detect such a difference (Pace et al., 1981; Foote and Kaproth, 1997). The present studies confirm that frozen semen programs using whole milk extender can be conveniently arranged with considerable latitude in the time sperm are held at 5°C before cryopreservation.

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REFERENCES


