EFFECT OF THAWING RATE AND POST-THAW TEMPERATURE ON MOTILITY AND ACROSOMAL MAINTENANCE IN BOVINE SEMEN FROZEN IN PLASTIC STRAWS

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SUMMARY

One ejaculate from each of 13 Holstein bulls was diluted in egg yolk-tris-glycerol, packaged in .25 ml Continental straws and frozen in liquid nitrogen vapor. Two thawing rates, 35 C for 1 min and 5 C for 3 min, were compared after immediate post-thaw exposure to either 1 C, 20 C or 37 C for either 1 or 3 minutes. Straws were plunged into water baths to achieve these thaw and post-thaw treatments. Following treatment, samples were incubated at 37 C and evaluated at 0 (immediately post-treatment), and again at 4 and 8 hr for percent intact acrosomes and percent motility. Time of exposure (1 vs 3 min) to the post-thaw temperature did not affect percent intact acrosomes. Considering mean percent intact acrosomes (mean of 0-, 4- and 8-hr incubation), the 35 C thaw resulted in significantly greater (P<.01) percent intact acrosomes after exposure to the 20 C and 37 C post-thaw treatments (47.1 and 48.8, respectively) than did the 5 C thaw (30.6 and 30.0, respectively). There was no difference in the mean percent intact acrosomes between the 5 C and 35 C thaws (37.6 vs 39.3, respectively) when exposed to the 1 C post-thaw treatment. For the 5 C thaw, there was a higher (P<.01) incidence of acrosomal damage when the semen was exposed immediately after thawing to 20 C or 37 C as compared to 1 C. A thaw rate x post-thaw treatment interaction was significant (P<.01) for percent intact acrosomes and percent motility. Results for motility were similar to those obtained for acrosomal maintenance.

(Key Words: Bovine Semen, Thawing Rates, Cold Shock.)

INTRODUCTION

The use of plastic straws for packaging frozen bovine semen has resulted in improved storage efficiency as well as recovery of more live spermatozoa and higher maintenance of the acrosome following the freeze-thaw process. Using a live-dead staining technique Aamdal and Anderson (1968) reported improvements in post-thaw recovery of live spermatozoa when .5 ml plastic straws were thawed rapidly. More recently, improvements in post-thaw motility (Robbins et al., 1972, 1973; Almquist and Wiggin, 1973) and post-thaw acrosomal maintenance (Robbins et al., 1972, 1973; Wiggin and Almquist, 1975) resulted when plastic straws were thawed rapidly.

While laboratory data clearly show that rapid thawing improves recovery of spermatozoa compared to slow thaws, the use of warm water and, thus, a warm semen sample has resulted in the fear that faster thaws may increase the likelihood of post-thaw cold-shock under field conditions. Critical laboratory data are not available relating thaw rate and post-thaw environment to post-thaw cell injury. Thus, the objective of this study was to compare motility and post-thaw acrosomal maintenance in bovine semen thawed at 5 C and 35 C followed by exposure to various post-thaw temperatures.

EXPERIMENTAL PROCEDURE

One ejaculate from each of 13 Holstein bulls was collected using an artificial vagina. Ejaculates were held at 37 C for 20 min before initial dilution in 50 ml of yolk-tris buffer containing fructose (fraction "A"). Penicillin-G and dihydrostreptomycin were present in the diluter at the level of 1,000 units and 1,000 µg per milliliter of final diluter. Flasks containing the partially diluted semen were placed into a beaker containing 250 ml of water at 37 C and...
were cooled to 5 C. Following a 2-hr cooling period additional fraction “A” was added to give a total sperm concentration of 160 x 10^6 cells/ml. Addition of an equal volume of yolk-tris containing 14% glycerol (fraction “B”) completed the dilution. Fraction “B” was added to fraction “A” in 10, 20, 30 and 40% increments (by volume) at 10-min intervals. Semen was packaged in .25 ml Continental straws and allowed to equilibrate for 4 hours.

After equilibration, straws were frozen in nitrogen vapor at -140 C. Upon reaching -100 C (approximately 7 min) the straws were plunged into liquid nitrogen and stored until used.

Semen thawed at 5 C/3 min or 35 C/1 min were exposed to the following treatments immediately after thawing: 1 C/1 min, 1 C/3 min, 20 C/1 min, 20 C/3 min, 37 C/1 min or 37 C/3 min. Thaws were accomplished by plunging five straws into a water bath maintained at 5 C or 35 C. Immediately after thawing, straws were plunged into water baths maintained at 1 C, 20 C or 37 C. After the post-thaw treatment, the contents of the straws were pooled into a 4-ml test tube and aliquots were evaluated for percent motility and percent intact acrosomes (0 hr). Samples were then placed into a 37 C water bath and subsequent evaluations were made at 4 and 8 hours. Percent motility was estimated at 37 C using phase microscopy. Percent intact acrosomes (percent cells with the apical ridge) were quantitated from direct counts of unfixed smears with a Zeiss differential interference contrast microscope (Saacke and Marshall, 1968; Saacke and White, 1972). All samples were coded to insure that the individual conducting the counts was not aware of the treatments being evaluated.

Thaw rate and the rate of post-thaw temperature changes were measured for each treatment using an ultra-miniature thermocouple (.2 mm). The thermocouple was securely placed in the center of the straw. Placement was made by drilling a hole (approximately .60 mm) in the plug of a previously thawed straw. Straws were then refrozen in liquid nitrogen, thawed (5 C or 35 C) and immediately exposed to the post-thaw treatments (1 C, 20 C or 37 C). Measurements at each thaw and post-thaw exposure were repeated 10 times.

Data were subjected to analyses of variance considering the five main effects and all possible two-way interactions. Selected means were subjected to further analysis using Duncan's Multiple Range Test.

RESULTS

There was significant variation (P<.01) due to thaw rates and ejaculates for both motility and acrosomal maintenance. Neither acrosomal maintenance nor percent motility varied significantly among post-thaw treatments of 1 C, 20 C and 37 C. Length of exposure (1 vs 3 min) to the post-thaw treatments did not affect acrosomal maintenance at any hour of evaluation. Therefore, means for the 1- and 3-min exposure periods for each post-thaw treatments were pooled for presenting the effect of thaw rate

<table>
<thead>
<tr>
<th>Thaw temperature (C)</th>
<th>Post-thaw treatment (C)</th>
<th>Mean percent intact acrosomes after incubation at 37 C for</th>
<th>Overall mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 hr</td>
<td>4 hr</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>62.6c</td>
<td>31.0b</td>
</tr>
<tr>
<td>35</td>
<td>1</td>
<td>60.1bc</td>
<td>34.8b</td>
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<tr>
<td>5</td>
<td>20</td>
<td>53.2a</td>
<td>23.9a</td>
</tr>
<tr>
<td>35</td>
<td>20</td>
<td>64.9cd</td>
<td>46.4c</td>
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<tr>
<td>5</td>
<td>37</td>
<td>55.7ab</td>
<td>20.4a</td>
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<tr>
<td>35</td>
<td>37</td>
<td>69.2d</td>
<td>45.2c</td>
</tr>
<tr>
<td>Error mean square</td>
<td></td>
<td>54.78 (86)c</td>
<td>48.56 (86)c</td>
</tr>
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</table>

a,b,c,d Different superscripts (by treatment) designate significant differences (P<.01) as tested by Duncan's Multiple Range Test.

e Degrees of freedom of the error mean square.
and post-thaw treatments on acrosomal maintenance and motility (tables 1 and 2). At all hours of evaluation there was a significant (P<.01) thaw rate x post-thaw treatment interaction for both acrosomal maintenance and percent motility. For the 1 C post-thaw treatment, percent intact acrosomes did not differ between thaws of 5 C or 35 C. However, semen thawed at 35 C had significantly greater (P<.01) percent intact acrosomes following exposure to the 20 C and 37 C post-thaw treatments when compared to semen thawed at 5 C. Unexpectedly, semen thawed at 5 C was significantly damaged (P<.01) following immediate exposure to either 20 C or 37 C as compared to the 1 C exposure. While more severe in some ejaculates, this acrosomal damage resulting from sudden temperature increases occurred in all 13 ejaculates.

Means for percent motility (table 2) represent values pooled for 1- and 3-min post-thaw time periods. Few differences in motility (P<.01) were present at 0 hour. However, at the 4-hr evaluation period the 35 C thaw exposed to 20 C and 37 C had significantly greater (P<.01) percent motile spermatozoa than the other thaw and post-thaw treatments (table 2). All treatments had very low motility after 8 hr of incubation. Therefore, little emphasis can be placed on differences due to treatment or thaw rates at the 8-hr evaluation period. Atypical motility patterns, often associated with cold shock, were not observed.

For both thaw rates the terminal temperature of the semen within the straw was that of the water bath used for thawing (5 C and 35 C, respectively). Thaw curves and temperature changes within the straw during the post-thaw treatments are presented in figures 1 and 2.

Discussion

Results of this study agree with previous thaw rate studies (Robbins et al., 1972, 1973; Almquist and Wiggins, 1973; Wiggins and Almquist, 1975) demonstrating a benefit to post-thaw acrosomal retention and motility following rapid thawing. However, the relationship of thaw rate to post-thaw environment, especially cold environments, has not been critically studied in frozen bovine semen. In undiluted semen the deleterious effect of sudden decreases in temperature (cold shock) has been well documented (Blackshaw, 1954; Blackshaw and Salisbury, 1957; Mann and Lutwak-Mann, 1955; Walton, 1957; Wales and White, 1959; White and Wales, 1961; Choong and Wales, 1962). However, the dramatic effects of cold shock on raw semen have not been clearly

Figure 1. Temperature changes within the straw during the 35 C thaw and during immediate post-thaw exposure to 1 C, 20 C and 37 C (10 replications).
demonstrated in diluted semen thawed at rapid rates. The relationship of thaw rate to post-thaw cold shock of semen packaged in the straw has been a topic of much speculation. Slow thaws (1 C to 5 C) with a low terminal post-thaw temperature are not susceptible to a sudden temperature decline and should be more resistant to cold shock. On the other hand, rapid thaws with end point temperatures approaching body temperature may be susceptible to a wide range of cold post-thaw temperatures thus increasing the likelihood of post-thaw cold shock. Such a relationship was not found in this study.

The rate of temperature decline (Mann and Lutwak-Mann, 1955) and the terminal temperature following a temperature drop (Wales and White, 1959; White and Wales, 1961) have been shown to influence the magnitude of damage due to cold shock in raw semen. Present results indicate that the same trend occurred in semen thawed at 35 C, but to a lesser degree. Damage to the acrosome and reduction in sperm motility resulted only when semen thawed at 35 C was exposed to the most severe cold shock treatment (1 C). When semen thawed at 35 C was suddenly exposed to 20 C, damage did not occur. Based on these results it appears that the diluent components of egg yolk and glycerol may provide post-thaw protection against all but extreme cold post-thaw temperatures.

Conventionally, increases in semen temperature within physiologic limits have not been associated with damage to spermatozoa. However, in this study, sudden exposure to either 20 C or 37 C after thawing at 5 C resulted in significant (<.01) acrosomal damage and reduction in motility. After thawing, a group of five straws was exposed directly to the post-thaw water baths prior to pooling the semen in a test tube. As a result, each straw within the group was subjected to sudden increases in temperature. Pooling cold semen before incubation at 37 C allows warming to occur gradually. In previous work testing thaw rates (Robbins et al., 1972; Robbins et al., 1973; Almquist and Wiggins, 1973; Wiggins and Almquist, 1975) samples pooled from several straws were allowed to warm gradually and thus no damage due to warming of the semen thawed at slow rates was detected. It appears from the present study that sudden post-thaw temperature changes in either direction will result in damage to spermatozoa. However, in light of the cell damage inherent to slow thaws (Robbins et al., 1972, 1973) it appears that slow thaw rates may increase the likelihood of post-thaw damage when sudden warming of the semen occurs. Such a possibility would exist particularly if cold semen were suddenly exposed to warm inseminating equipment. The fact that significant damage (P<.01) occurred due to sudden post-thaw temperature increases (20 C and 37 C) following a 5 C thaw further supports the need for rapid thaws of semen frozen in plastic straws.

LITERATURE CITED