Computer-Assisted Sperm Analysis for Assessing Initial Semen Quality and Changes During Storage at 5°C

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ABSTRACT

Computer-assisted sperm analysis equipment was used to evaluate bull sperm initially in a modified Tyrode’s solution, in Cornell University extender, and in egg yolk-glycerol-Tris extender (following cooling and storage in the latter two extenders). Two ejaculates of semen were collected from each of eight bulls. Semen was divided into aliquots using a factorial arrangement. The semen, diluted to approximately \(20 \times 10^6\) sperm/ml, was loaded into two 20-\(\mu\)m chambers, and six microscope fields from each chamber were videotaped for each treatment of each ejaculate of semen. Eight sperm characteristics analyzed with the Hamilton Thorne integrated visual optical system (Hamilton Thorne, Beverly, MA) were reported, and some of these characteristics differed significantly among bulls. The initial values for motile sperm in modified Tyrode’s solution, Cornell University extender, and egg yolk-glycerol-Tris extender were 87, 79, and 66%; little change followed cooling and storage at 5°C in the latter two extenders. Also, there was a small but significant decline in sperm velocity during 3 d of storage. Hyperactive sperm increased slightly during storage. The procedures used can rapidly and accurately measure many sperm characteristics in fresh semen and in semen stored in egg yolk extenders, and differences among bulls can be detected.

(Key words: bull sperm evaluation, egg yolk extenders)

Abbreviation key: CASA = computer-assisted sperm analysis, CUE = Cornell University extender, EYGT = egg yolk-glycerol-Tris extender.

INTRODUCTION

Tests of semen quality that are repeatable and are related to fertility and quality of semen are important, and many have been reported (4, 17, 19, 30, 31, 32). More recently, remarkable advances have been made in the evaluation of various sperm characteristics through computer-assisted sperm analysis (CASA). Sperm from different species have been examined by CASA (6, 7, 8, 9, 10, 16, 22). When sperm in semen that contains a variety of particles and debris are being evaluated, it is necessary to develop procedures to screen out, by optical means, all interfering material; such screening can be done to some extent by setting gates to exclude foreign objects that are similar in size to sperm.

To process semen for use in insemination, various protective agents such as egg yolk or milk are added to prevent cold shock, and glycerol is added to protect sperm during freezing (12, 13, 21). Although most bull sperm that is used for AI is cryopreserved, extenders for liquid semen are still used (23, 34). Some of the extenders add particulate material that has the potential to interfere with CASA. In the present paper, we report the first studies of an evaluation by CASA of fresh and cooled sperm in semen extenders that have been or are currently used widely for processing bull semen for AI.

MATERIALS AND METHODS

Bull Semen

Bull semen was obtained on a regular collection schedule from eight bulls on two occasions (16 ejaculates) according to the regular procedures at Genex Cooperative, Inc. (Ithaca, NY) (5). Semen from these bulls had been used to inseminate 456,000 cows prior to this study, and these inseminations were used to provide an estimate of the relative fertility of each bull. Each bull was carefully prepared sexually, and the semen was collected with an artificial vagina. The semen was weighed to calculate the volume of the ejaculate. Each ejaculate was maintained in a water bath at 35°C while the sperm concentration was estimated spectrophotometrically. The percentage of motile sperm was estimated subjectively (5) after dilution on a slide that was maintained at 37°C.
The initial concentration of sperm in each ejaculate was used to calculate the dilution of sperm so that all semen was processed for CASA with approximately $20 \times 10^6$ sperm/ml. Each sample of fresh semen at 35°C was divided into aliquots into TALP (Tyrode’s-albumin-lactate-pyruvate), as reported by Bavister and Yanagimachi (3) (Appendix Table A1), and also into Cornell University extender [CUE; (14, 15)] and egg yolk-glycerol-Tris extender [EYGT; (7, 11, 12)] at 35°C. Each 2.5-ml tube contained 2 ml of diluted semen. All buffers were prepared aseptically with milli-Q purified water and were sterilized by passing them through 0.25 μm-diameter Millipore filters (Millipore Filter Co., Bedford, MA). Analytical grade reagents were used to prepare the buffers, and 20% egg yolk (vol/vol) was added to make the CUE and EYGT extenders. The pH was 6.8.

Sperm in all three media were analyzed by CASA soon after collection. In addition, sperm in the CUE and EYGT extenders were examined subsequently. These samples were cooled from 35 to 5°C in water jackets. After cooling and during storage at 5°C for 3 d, the semen in CUE and in EYGT was mixed daily and examined by CASA.

### CASA

For all experiments, Microcell Chambers (20 μm deep; Conception Technologies, La Jolla, CA) were used with the Hamilton Thorne integrated visual optical system (version 10; Hamilton Thorne, Beverly, MA) operated at 37°C. Chambers on a slide warmer at 37°C were loaded with 7 μl of semen that were diluted to approximately $20 \times 10^6$ sperm/ml. Both chambers on each slide were used to perform duplicate determinations. Six fields of sperm per chamber were evaluated, omitting the first two fields at each end of the chamber, because unpublished observations (P. B. Farrell and R. H. Foote, 1993) had indicated that these fields tended to provide divergent results. These fields were recorded with a JVC Super Video Home Systems video recorder (JVC, Tokyo, Japan). Twelve characteristics of sperm motion were studied. Eight traits that appeared to be most useful in characterizing sperm were presented, and these traits are defined in the results. Instrument settings are given in Table 1.

Many fields were examined visually to check that sperm were being appropriately classified by the analyzer. This classification was especially important to ensure proper identification of the hyperactive sperm, which swim in a star-shaped pattern.

### Statistical Analysis

Two analyses of variance were performed. The first compared the three media used for examination of fresh semen. The model was $y_{ijk} = \mu + a_i + b_j + a_{ij} + e_k$, where $a_i$ is random effect of eight bulls, $b_j$ is fixed effect of three media, $a_{ij}$ is $a_i \times b_j$ interaction, and $e_k$ is error term. The second analysis was similar; it considered eight bulls, the fixed effects of five time intervals and two extenders, the three first-order interactions, and the error term, which was composed of the second-order interaction. The 80 $(8 \times 5 \times 2)$ individual observations for each trait were the pooled result of examining sperm in 12 fields (two chambers with six fields per chamber). Data for the two evaluates for each bull were averaged. Orthogonal contrasts were used to make individual comparisons, and differences at $P \leq 0.05$ were considered to be statistically significant.

### RESULTS

Means for eight variables are presented in Table 2. Data in the first four columns represent sperm swimming speed, which provide a measure of the curvilinearity of trajectory. For example, straightness is a ratio of the straight line distance between the starting point and endpoint of a sperm track compared
with the smoothed path traveled by the sperm cell, expressed as a percentage. The smaller percentages represent a more circular route traveled, and, although the values in Table 2 are calculated from individual data points, the straightness percentage values approximate straight line velocity and average path velocity. The analyses of variance for these data are presented in Tables 3 and 4.

### Fresh Semen

Bulls differed for most variables except for the percentage of motile sperm. One of the largest differences was in critical velocity. The mean critical velocity and the range in TALP was 208 (177 to 235) μm/s, in CUE was 181 (169 to 200) μm/s, and in EYGT was 147 (125 to 174) μm/s. Bulls ranked nearly the same in all media, which is reflected in the nonsignificant (P = 0.70) interaction of bull and media (Table 3). Media effects were the large sources of variation (Table 3) as reflected by the mean differences (Table 2). Interactions of media and storage were small.

### Cooled and Stored Semen

Media type was again the largest source of variation for most variables. Cooling and storage had substantial effects, as did bulls (Table 4). These differences between media and small decreases in most velocity measurements of sperm over time along with other variables are summarized in Table 2.

Velocity measurements such as average path velocity and critical velocity increased after sperm were cooled (P < 0.05) and then declined slightly during storage. The lateral head movement increased conspicuously after sperm were cooled in CUE and in EYGT (Table 2), resulting in a large storage effect (Table 4). Although sperm swam more slowly in EYGT than in CUE, the substantial lateral beat of the tail was detectable by visual observation. There was little change within media in the percentage of motile sperm as a result of cooling or storage for 3 d. However, a higher percentage of motile sperm was maintained in CUE than in EYGT.

As with the fresh semen, interactions generally were small, although some were statistically significant. Sperm from some bulls tended to swim in more circular patterns in EYGT than in CUE, accounting for the substantial interaction of bull and medium for straightness (P < 0.01). These bulls tended to have fewer motile sperm in EYGT also, accounting for part of the interaction of bull and medium (P < 0.01) for motility.
TABLE 3. Analysis of variance of measurements on fresh semen. 1

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>VAP 2</th>
<th>VSL</th>
<th>STR</th>
<th>VCL</th>
<th>ALH</th>
<th>CONC</th>
<th>MOT</th>
<th>HYP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bull (B)</td>
<td>7</td>
<td>212 &lt;0.01</td>
<td>166 &lt;0.08</td>
<td>24.3 &lt;0.01</td>
<td>1098 &lt;0.01</td>
<td>1.7 &lt;0.01</td>
<td>130 &lt;0.01</td>
<td>51 0.27</td>
<td>3.6 0.05</td>
</tr>
<tr>
<td>Media (M)</td>
<td>2</td>
<td>8600 &lt;0.01</td>
<td>8029 &lt;0.01</td>
<td>74.5 &lt;0.01</td>
<td>15220 &lt;0.01</td>
<td>1.2 0.16</td>
<td>244 &lt;0.01</td>
<td>1682 &lt;0.01</td>
<td>7.0 &lt;0.05</td>
</tr>
<tr>
<td>B × M</td>
<td>14</td>
<td>38 0.93</td>
<td>42 0.89</td>
<td>3.9 0.86</td>
<td>163 0.70</td>
<td>0.6 0.20</td>
<td>20 0.82</td>
<td>31 0.66</td>
<td>1.8 0.38</td>
</tr>
<tr>
<td>Error</td>
<td>24</td>
<td>83 ...</td>
<td>78 ...</td>
<td>6.9 ...</td>
<td>217 ...</td>
<td>0.4 ...</td>
<td>33 ...</td>
<td>38 ...</td>
<td>1.6 ...</td>
</tr>
</tbody>
</table>

1The mean squares (MS) have been rounded off to fit in the table.

2VAP = Average path velocity (micrometers per second), VSL = straight line velocity or progressive velocity (micrometers per second), STR = straightness (percentage of VSL/VAP), VCL = critical velocity or track speed (micrometers per second), ALH = amplitude of lateral head displacement (microns), CONC = cell concentration (10^6 ml), MOT = motile sperm (percentage), and HYP = hyperactive sperm (percentage). Hyperactive sperm swim in a star-like pattern.

DISCUSSION

The development of CASA has made possible the measurement of many sperm characteristics rapidly, objectively, and with an adequate number of sperm to provide sensitive assessment (2, 6, 9, 16, 22, 24, 25, 26, 28, 29, 36, 37). The validity of the results depends on careful sample preparation and the settings used on the instrument (2, 23) to identify correctly moving sperm, nonmoving sperm, and particles (usually static) other than sperm. The Hamilton Thorne equipment permits the user to alter the settings easily, and, by visual inspection of the screen, to verify whether or not the settings result in correct particle discrimination. This inspection was conducted in the present study for each type of media used to dilute or extend the sperm.

In this study, TALP was chosen as an optically clear medium that is commonly available in laboratories. This medium supports good sperm motility in several species (3, 10). The CUE was chosen because it was one of the most, if not the most, successful extender used for unfrozen bull sperm (14, 15), and a Cornell University 16 modification (15) led to the development of Caprogen, which is used in New Zealand for most of the AI of dairy cattle with unfrozen semen (34, 35). The EYGT was chosen because it is a successful extending medium for both liquid and frozen bull sperm (7, 11, 12) and is the most widely used extender for freezing bull semen worldwide (21).

No studies were found that reported sperm changes in these extenders following cooling and storage of sperm, although several studies have explored using CASA to evaluate bull sperm under a variety of conditions (1, 2, 6, 18, 20, 25, 26, 36). The bull semen was collected from a cross-section of Holstein bulls used extensively in AI. Therefore, the values obtained should be typical of values for semen from a breed of bulls that is widely used for AI. Values were different from those obtained for sperm that were incubated in media specially designed to induce capacitation (1, 18, 20, 25, 26).

The settings given in Table 1 to obtain these data also should be useful for anyone operating the Hamilton Thorne integrated visual optical system. During our experiment, the screen was periodically checked.

TABLE 4. Analysis of variance of measurements on cooled and stored semen.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>VAP 2</th>
<th>VSL</th>
<th>STR</th>
<th>VCL</th>
<th>ALH</th>
<th>CONC</th>
<th>MOT</th>
<th>HYP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bull (B)</td>
<td>7</td>
<td>130 &lt;0.01</td>
<td>100 &lt;0.01</td>
<td>76.0 &lt;0.01</td>
<td>967 &lt;0.01</td>
<td>3.93 &lt;0.01</td>
<td>55.5 &lt;0.01</td>
<td>28.3 0.06</td>
<td>20.0 &lt;0.01</td>
</tr>
<tr>
<td>Media (M)</td>
<td>1</td>
<td>11,899 &lt;0.01</td>
<td>12,148 &lt;0.01</td>
<td>652.2 &lt;0.01</td>
<td>30,840 &lt;0.01</td>
<td>0.11 0.78</td>
<td>1061.9 &lt;0.01</td>
<td>2649.3 &lt;0.01</td>
<td>149.6 &lt;0.01</td>
</tr>
<tr>
<td>Storage (S)</td>
<td>4</td>
<td>591 &lt;0.01</td>
<td>1878 &lt;0.01</td>
<td>835.3 &lt;0.01</td>
<td>3640 &lt;0.01</td>
<td>25.75 &lt;0.01</td>
<td>8.9 0.86</td>
<td>34.3 0.06</td>
<td>42.3 &lt;0.01</td>
</tr>
<tr>
<td>B × M</td>
<td>7</td>
<td>43 0.09</td>
<td>25 0.04</td>
<td>41.2 &lt;0.01</td>
<td>406 0.02</td>
<td>1.33 &lt;0.01</td>
<td>14.7 0.17</td>
<td>42.8 0.01</td>
<td>7.6 0.01</td>
</tr>
<tr>
<td>B × S</td>
<td>28</td>
<td>32 0.16</td>
<td>46 &lt;0.01</td>
<td>18.4 0.03</td>
<td>156 0.44</td>
<td>0.48 0.06</td>
<td>27.9 &lt;0.01</td>
<td>13.2 0.46</td>
<td>4.4 0.07</td>
</tr>
<tr>
<td>M × S</td>
<td>4</td>
<td>136 &lt;0.01</td>
<td>143 &lt;0.01</td>
<td>4.6 0.73</td>
<td>334 0.09</td>
<td>0.37 0.26</td>
<td>68.6 &lt;0.01</td>
<td>5.6 0.78</td>
<td>4.3 0.17</td>
</tr>
<tr>
<td>Error</td>
<td>28</td>
<td>21 ...</td>
<td>10 ...</td>
<td>9.0 ...</td>
<td>148 ...</td>
<td>0.26 ...</td>
<td>9.0 ...</td>
<td>12.9 ...</td>
<td>2.5 ...</td>
</tr>
</tbody>
</table>

1VAP = Average path velocity (micrometers per second), VSL = straight line velocity or progressive velocity (micrometers per second), STR = straightness (percentage of VSL/VAP), VCL = critical velocity or track speed (micrometers per second), ALH = amplitude of lateral head displacement (microns), CONC = cell concentration (10^6 ml), MOT = motile sperm (percentage), and HYP = hyperactive sperm (percentage). Hyperactive sperm swim in a star-like pattern.
to verify that objects were classified correctly by CASA, and a few particles in EYGT were identified as static sperm. This classification resulted in a higher sperm count than the planned $20 \times 10^6$/ml.

**Fresh Semen**

Sperm from different bulls differed for many traits when evaluated by CASA across the three media. Because interactions of bull and media generally were small indicates that all three media may give comparable results. However, TALP is widely available, promotes high sperm motility, and is a particle-free medium that has low viscosity. Therefore, TALP has several advantages for routine evaluation of fresh semen. If sperm are to be cooled and stored at 5°C, then protective substances such as egg yolk are required.

For stored semen, CUE has an advantage in that the velocity of the sperm cells and the percentage of motile sperm are higher in CUE than in EYGT. Thus, with this greater range from zero, there is a higher probability of detecting true differences among bulls when using CUE than when using EYGT, which tended to be true for the voluminous data collected (data not shown). However, differences in semen among bulls tended to be small as a result of the selection of the bulls that survive the various culling programs in AI.

Fertility data on these 16 ejaculates were not available to permit comparison of semen quality. However, the previously accumulated information on nonreturn rate of these bulls was extensive and ranged from 66 to 72%; for 5 of the bulls, nonreturn rate ranged from 69 to 71%. Correlations calculated between the estimated fertility of these bulls and individual traits of semen quality averaged over two ejaculates were not significant ($P > 0.05$). This result was expected because of the narrow range of fertility values. How semen from bulls with lower fertility would respond is uncertain.

**Cooled and Stored Semen**

Bull effects were significant for several traits measured on cooled and stored semen (Table 4). However, as with fresh semen, none of the traits were correlated with fertility. This result is not surprising because of the uniformity among bulls and because fertility was based entirely on inseminations with frozen semen. There were some significant interactions, of bulls with media and storage, but these interactions were generally small, and some were due to chance because of the multiple combinations calculated.

The overall measurements on stored sperm in CUE tended to reflect a higher quality than those in EYGT ($P < 0.05$). However, both resulted in high fertility when comparable low numbers of sperm were inseminated (11, 15). This result was interpreted to mean that, if minimum standards are set for the semen from different bulls, the standards should be for a specific extender. This inference also is supported by the several interactions of bulls and media (Table 4).

The cooling and storage effects are particularly interesting. In both CUE and EYGT, sperm velocity and the percentage of motile sperm were higher ($P < 0.05$) after cooling than before cooling, which suggests that there was an adjustment or accommodation of the sperm cells to the two media containing egg yolk during this cooling period. All subsequent measurements over 3 d revealed little change or a modest decline. For example, the straight line velocity of the sperm decreased by d 3, and the percentage of motile sperm changed little. When semen was preserved with CUE for breeding a large number of cows with liquid semen (14, 15), fertility changed little for 2 d but then declined by 3 to 4 percentage points during 3 to 4 d of storage. The results (Table 2) suggest that velocity measurements might be more useful than the percentage of motile sperm in predicting the fertility of bull sperm stored in various media. The survival of sperm in vitro generally has been reported to exceed the duration of its high fertilizing ability (33, 35).

Lateral head movement increased in both extenders after cooling for reasons that are not clear. The pattern of sperm swimming was different from cold shocked sperm. Furthermore, sperm were cooled slowly using the standard procedure that had previously resulted in high fertility (11, 14, 15).

Sperm concentration in EYGT tended to be overestimated throughout, as there was some particulate matter in this medium that occasionally was identified by CASA as static cells. Thus, the percentage of motile cells would also be underestimated. Visual observations of this phenomenon were not sufficient to make a correction.

The decline in concentration of sperm cells during storage in CUE is an enigma. R. H. Foote (1960, unpublished data) has observed sperm sticking to container walls, and it is possible that more sperm adhered to the containers as storage time increased with CUE.

Hyperactivate sperm swim in exaggerated lateral movements forming a star-shaped pattern (27). This pattern is associated with capacitation under a variety of incubation conditions (1, 18, 20, 25, 26, 27), but little hyperactivation of this type would be expected at 5°C. In EYGT, the proportion of hyperactive sperm was higher than that in CUE ($P < 0.01$), but
the percentage was low. Because the increase occurred by the time sperm were cooled and because there was no increase with storage, the hyperactivation observed was probably not associated with the typical tendency of the sperm to undergo the acrosome reaction. Rather, this result was more likely some ionic response of a few cells to the Tris molecule.

CONCLUSIONS

The present study characterized for the first time a variety of sperm movements that are measurable by CASA when bull sperm were evaluated initially, after cooling, and after storage in media that were commonly used for preservation of unfrozen bull sperm. Instrument settings for correctly identifying motile and nonmotile sperm and static particles under these conditions are given. The high velocity of movement by the high percentage of motile sperm maintained during storage at 5°C for 2 d was consistent with the high fertility obtained previously when CUE and EYGT were used to preserve unfrozen semen.

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REFERENCES

1 Anderson, S. H., and G. J. Killian. 1994. Effect of macro-
13 Foote, R. H., and R. W. Bratton. 1949. The fertility of bovine semen cooled with and without the addition of citrate-
sulfanilamide-yolk extender. J. Dairy Sci. 32:856.
14 Foote, R. H., and H. O. Dunn. 1962. Motility and fertility of bull semen extended at high rates in yolk extender containing cata-
nation. Gráficas Orde, Madrid, Spain I-115.
24 Liu, D. Y., G. N. Clarke, and H.W.G. Baker. 1991. Relationship between sperm motility assessed with the Hamilton-Thorn motil-
26 McNutt, T. L., P. Olds-Clarke, A. L. Way, S. S. Saurez, and G. J. Killian. 1994. Effect of follicular or oviductal fluids on move-
drol. 11:195.
28 O'Connor, M. T., R. P. Amann, and R. G. Saacke. 1981. Compar-
TABLE A1. Composition of TALP (Tyrode’s-albumin-lactate-pyruvate).\textsuperscript{1}

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrode’s solution (stock for making TALP)</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>5.69</td>
</tr>
<tr>
<td>KCl</td>
<td>0.23</td>
</tr>
<tr>
<td>NaHPO₄</td>
<td>0.04</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.09</td>
</tr>
<tr>
<td>MgCl₂·H₂O</td>
<td>0.08</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>0.29</td>
</tr>
<tr>
<td>Milli-Q\textsuperscript{2} Water to 1000 ml</td>
<td></td>
</tr>
<tr>
<td>TALP (added to Tyrode’s solution)</td>
<td></td>
</tr>
<tr>
<td>Na Pyruvate</td>
<td>0.02</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.90</td>
</tr>
<tr>
<td>Lactic acid, ml</td>
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</tr>
<tr>
<td>HEPES</td>
<td>2.38</td>
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<tr>
<td>BSA</td>
<td>6.00</td>
</tr>
<tr>
<td>Penicillin, U</td>
<td>20,000</td>
</tr>
</tbody>
</table>

\textsuperscript{1}pH = 6.8.

\textsuperscript{2}Millipore Filter Co. (Bedford, MA).