Sizing of Aging Bull Spermatozoa with an Electronic Counter

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Abstract

Cell size was evaluated as a criterion of the functional state of spermatozoa. Packed cell volume of 13 samples of bull semen stored at room temperature for 1, 3, 5, 7, 9 and 11.5 hours averaged 8.7, 10.6, 11.2, 12.0, 12.1, and 12.2%. Corresponding mean window values obtained with a Coulter Counter were 12.3, 14.3, 15.6, 16.0, 16.1, and 15.9. Correlations between these two types of measurements ranged from 0.87 to 0.99 (P < 0.01) for 11 of the 13 bulls. Spermatozoa killed by freezing and thawing did not swell. Mixtures of live and dead spermatozoa stored at 7°C show a bimodal distribution formed by the two types of cells. Following death of all cells, a single distribution remained. In a study of size changes during storage of 87 ejaculates from six bulls, bulls were found to differ (P < 0.05). Samples high in sperm concentration swelled less and for a shorter period; motility declined more rapidly during storage. Size changes measured in living cells with this technique offer an approach to studying maintenance of integrity of the cell in a variety of environments.

Measurement of sperm cell size in mammals has been directed particularly toward determining whether gametes formed in the heterogametic sex showed a bimodal distribution of sizes. While studying packed cell volume (3), it was observed that the absolute volume appeared to be influenced by the time that elapsed from collection to processing the sample, thus suggesting an aging effect. Drevius and Eriksson (2) have shown that spermatozoa can undergo osmotic swelling. It has been clearly demonstrated that the size of a variety of microscopic structures (1, 9, 13, 15) including spermatozoa (5-8, 10, 11, 14) can be detected by electronic particle sizing equipment.

In a previous report from this laboratory (4) it was shown that size changes in spermatozoa during aging may be detected with such equipment. The present paper is a more extensive report of this phenomenon.

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Experimental Procedure

Bull spermatozoa were obtained from Eastern Artificial Insemination Cooperative, Inc. Packed cell volume was determined after centrifuging for 10 minutes at 12,500 rpm, as described (3). The size of spermatozoa also was measured with a Coulter Counter, Model B, and an attached X-Y plotter. A tube with an aperture 50 μ in diameter was used. The instrument works on the principle that particles suspended in an electrolyte and aspirated through an aperture conducting an electrolytic current alter the electrical resistance as they pass through. If particle counting rate is appropriate so that coincidence effects are negligible, the change in resistance is proportional to particle size (9, 13).

Spermatozoa were suspended in 0.87% w/v sodium chloride solution at a dilution of about 1:10,000 just before electronic sizing. The saline solution was filtered through a Millipore filter (0.45 μ pore diameter) before use, and all glassware washed with glass redistilled water similarly filtered to remove extraneous particulate matter.

Several thousand sperm cells can be counted and sized per second, so the statistical accuracy is high. Correlation coefficients calculated for a series of semen samples counted in duplicate consistently were ≥0.99, in general agreement with other work (6, 10, 12).

The size analyzer was calibrated using latex spheres with a diameter of 2.956 ± 0.015 μ and a volume of 13.52 μ³. However, the size changes are reported on a relative basis because of the uncertainty that a spermatozoon would give precisely the same value as a spherical particle of identical volume.

An experiment was conducted to study the effect of age of spermatozoa at the time of ejaculation and the effect of in vitro storage on cell volume. Six mature bulls were ejaculated in a changeover design four times per week for four weeks versus one time per week for four weeks. The order of treatment was reversed for half of the bulls. Following semen collection, spermatozoa were stored undiluted at room temperature for 1, 3, 5, 7, 9, and 11.5 hours, and cell size was monitored electronically on 87 ejaculates.

The size frequency distributions were re-
Results and Discussion

Thirteen ejaculates of semen stored at room temperature for 1, 3, 5, 7, 9, and 11.5 hours had mean spermatocrit values (per cent cells) of 8.7, 10.6, 11.2, 12.0, 12.1, and 12.2%, respectively. Corresponding mean windows in the electronic sizer were 12.3, 14.3, 15.0, 15.5, 16.2, and 16.3. The marked change with time is shown graphically in Fig. 1. When the paired values for each of the 13 ejaculates at the different time intervals were correlated, 11 out of 13 correlation coefficients ranged from 0.87 to 0.99 (P < 0.01). Two lower values (0.56 and 0.64) were obtained for ejaculates that showed little or erratic swelling response without an obvious reason.

The results of electronic sizing 13 to 15 ejaculates per bull (87 ejaculates total) following storage as undiluted semen at room temperature are shown in Table 1. Bulls were significantly different (P < 0.05), and spermatozoa from certain bulls increased in size more during storage than spermatozoa from other bulls.

A separate distribution of smaller particles was observed in the counter which was excluded in these studies of sperm cell volume. Microscopic examination of these particles isolated by centrifugation revealed they were the protoplasmic droplets. When undiluted semen was stored at 7°C or frozen and thawed, the droplet distribution disappeared.

The analysis of variance showing the statistical significance of the bull effects and the effects of frequency of semen collection are given in Table 2. The effect of collection frequency became more pronounced after 7 hours of storage (P < 0.01).

Frequent ejaculation tended to reduce concentration of spermatozoa per milliliter of semen (r = -0.72). When semen samples were divided into an upper and lower half based on sperm concentration, the swelling patterns for the lower half were similar to the four times per week series and the upper half similar to the one time per week series. These similarities are shown in Table 3. The mean sperm concentration for the upper half was $1,981 \times 10^8$/milliliter, and for the lower half it was $970 \times 10^6$/milliliter. The freezing point depression for the seminal plasma of both groups was 0.55°C.

### Table 1. Volume changes in spermatozoa during storage as measured electronically.

<table>
<thead>
<tr>
<th>Storage time (hr)</th>
<th>Bulls</th>
<th>1 3 5 7 9 11.5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10.2</td>
<td>10.2</td>
</tr>
<tr>
<td>Frequency of collection (F)</td>
<td>0.9</td>
<td>2.4</td>
</tr>
<tr>
<td>B × F</td>
<td>0.7</td>
<td>0.9</td>
</tr>
</tbody>
</table>

a = P < 0.05; b = P < 0.01.

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The effect of sperm concentration on size changes is shown graphically in Fig. 2. When sperm concentration was held constant by covariance analysis, frequency of semen collection was without effect on swelling. Thus, the frequency of collection effect seems to be primarily a sperm concentration effect. Fig. 2 reveals further that motility was associated with the concentration effect. Spermatozoa in the less concentrated semen samples remained motile longer and increased in mean size 46% vs. 24% for the more concentrated samples. Motility was more oscillatory than progressive at these high dilutions. Often spermatozoa with curved tails moving in circles were observed during the swelling phase. No metabolic studies were conducted. However, the effect of concentration on motility and swelling was noticeable only after several hours of storage, and it is likely that the more concentrated samples produced more lactic acid and exhausted the available fructose sooner.

That no differences were seen initially indicates that the frequent semen collections did not hasten sperm transport through the epididymides sufficiently to cause any difference in initial cell size. Furthermore, that there were no marked effects during storage associated with frequent ejaculation, other than the sperm concentration effect, suggests that spermatozoa obtained by the two treatments were similar in swelling behavior.

In examining other frequency distributions of spermatozoa stored at 7°C and later at room temperature, it was observed that only a portion swelled. This resulted in a bimodal distribution, with the smaller cells corresponding in volume to dead cells. Spermatozoa killed by quick freezing and thawing did not undergo any size increases measured by the electronic sizer or by the spermatocrit technique. Samples that showed an unexplained early loss of motility during room temperature storage also did not swell normally. Furthermore, when spermatozoa were stored until dead they returned to their original size. These results appear to confirm the expectation that only the live spermatozoa undergo swelling.

Any conditions studied which hastened death caused cells to return to their original size more rapidly. Furthermore, other observations revealed that spermatozoa extended and stored in buffered media favoring prolongation of motility also delayed the onset of swelling until motility ceased. Hypertonic media caused a small reduction in the mean window and spermatocrit values. Hypotonic media had the opposite effect when there were live spermatozoa present. The latter treatment produced a bimodal distribution of swollen live cells and smaller lysed cells. These findings are consistent with reports for other cell types (15), and emphasize size as an important possible criterion of the functional state of spermatozoa.

With the settings used in the present experiments the mean window after one hour was 12.3, and this represented a mean volume of 23.7 μm³. Dead and unswellen spermatozoa appeared to be slightly smaller than this, which is in agreement with other reports on volume of bull spermatozoa (6, 11). On the other hand, some spermatozoa were estimated to have

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**Table 3. Association of collection frequency and sperm concentration.**

<table>
<thead>
<tr>
<th>Volume (mean window)</th>
<th>Storage time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  3  5  7  9  11.5</td>
</tr>
<tr>
<td>Collection frequency</td>
<td></td>
</tr>
<tr>
<td>Four times/week</td>
<td>12.4 14.3</td>
</tr>
<tr>
<td>One time/week</td>
<td>12.2 14.6</td>
</tr>
<tr>
<td>Sperm concentration</td>
<td></td>
</tr>
<tr>
<td>Lower half</td>
<td>12.3 14.2</td>
</tr>
<tr>
<td>Upper half</td>
<td>12.3 14.5</td>
</tr>
</tbody>
</table>

**Fig. 2. Changes in sperm cell size in ejaculates with low and high concentrations of spermatozoa and the associated proportions of motile cells.**
swollen to three times this mean volume. Although they did not reveal such an increase when observed with ordinary light microscopy, this finding is in good agreement with the osmotic swelling reported by Drevius and Eriksson (2), who utilized phase and electron microscopy. Presumably, area measurements (17) under these conditions are not a good indicator of total volume. An additional advantage of the electronic sizer is the high rate of speed with which spermatozoa are sized without disrupting the physiological condition of spermatozoa during measurement. This technique would appear to hold considerable promise for the assay of the effect of a variety of treatments on the maintenance of the functional integrity of the sperm cell. Its application to bull spermatozoa stored under a variety of carefully defined experimental conditions will be reported subsequently.

Acknowledgments

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References