FLOW CYTOMETRIC DETERMINATION OF THE PROPORTIONS
OF X- AND Y-CHROMOSOME-BEARING SPERM IN SAMPLES
OF PURPORTEDLY SEPARATED BULL SPERM

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Summary

A rapid assay for determining the proportions of X- and Y-chromosome-bearing sperm in semen samples would benefit research aimed at sex ratio control through sperm separation. It would also be of value for quality control should a separation technique be developed. Flow cytometric methods capable of measuring sperm DNA content precisely enough to resolve and quantify the X and Y populations in many mammalian species have been developed. They are effective for fresh and cryopreserved sperm of most domestic animals. Results are reported of flow cytometric analyses of bull sperm samples from seven commercial and academic sources after processing with procedures purported to separate the X and Y populations. In no case was enrichment of either sperm population observed. Breeding trials carried out by the sources of two of the sets of samples showed these procedures were ineffective in altering the sex ratio.

(Key Words: Sperm Separation, Sex Ratio, Flow Cytometry, Sperm Deoxyribonucleic Acid.)

Introduction

Flow cytometric techniques have been developed that are capable of measuring the DNA content of individual sperm with sufficient accuracy to resolve the two sex-determining populations in several mammalian species, including those of agricultural interest. Measurements can be made on fresh or cryopreserved semen (Pinkel et al., 1982b; Garner et al., 1983). These measurements have been successful for human sperm (Otto et al., 1979), but are not repeatable. Limited numbers of nonviable, separated sperm have been isolated using cell sorting in conjunction with flow cytometry (Pinkel et al., 1982a). Here we report the flow cytometric determination of...
the proportions of X- and Y-chromosome-bearing sperm in bull semen treated with various techniques purported to separate the two populations. The semen samples and appropriate controls were furnished by seven commercial and academic sources.

**Experimental Procedures**

*Semence Preparation.* Bovine semen samples, which had been purportedly enriched in either X or Y sperm, were analyzed at the request of the suppliers. These samples had been processed using enrichment techniques that included: (1) convection counter streaming within a charged field; (2) albumin gradient systems, (3) other density gradient-chemical systems and (4) immunologic affinity to a sex-specific antigen. The sperm were provided either fresh in extenders, cryopreserved in straws, or fixed in 80% ethanol after washing. Preparation for analysis is described by Garner et al. (1983). Briefly, the fresh and thawed samples were washed with a series of sodium citrate-dimethyl sulfoxide (DMSO) solutions in which the DMSO concentration increased from 5 to 50%. The cells then were fixed in 80% ethanol for at least 1 h before staining. For DNA staining, an aliquot of $1 \times 10^7$ fixed sperm was incubated in a papain-dithioerythritol solution to partially decondense the chromatin to allow stain penetration. The tails and most of the membranes and cytoplasm also were removed (Garner et al., 1984-). Sperm DNA was stained with 20 nM 4'-6-diamidino-2-phenylindole (DAPI). Stain uptake reached equilibrium in less than 5 min; samples were stable at room temperature for more than 24 h and for many weeks at 4 C.

*Instrumentation.* The suspensions of stained sperm nuclei were measured in an ICP22 flow cytometer7. This instrument is an epillumination fluorescence microscope focused into a flow chamber (figure 1). Fluorescence was excited with a mercury lamp. Cells flowed toward the objective lens along the optical axis at a rate of 300 to 500/s. Just after passing through the focal plane, where their fluorescence was measured, they turned 90° and were swept to a waste container. This instrument was used because its measurement geometry overcomes optical difficulties in sperm DNA content measurement (Gledhill et al., 1976; Pinkel et al., 1982b). The fluorescence emitted from each nucleus was converted to an electrical pulse by a photomultiplier tube, the height of each pulse being proportional to the stain content and thus the amount of DNA. The distribution of pulse heights was recorded with a multichannel analyzer. Simulated distributions are shown in figure 2 as they would appear for samples with different proportions of the two sperm populations. The presence of enrichment would be visually striking. Quantitative analysis of experimental distributions was done by a computer program that fits the sum of two Gaussian (normal) curves to the observed data (figure 3) by adjusting their means, areas and

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7 Ortho Instruments Westwood MA.
Figure 2. Data from the flow cytometer is presented as a frequency distribution showing the number of sperm nuclei vs fluorescence intensity. Two peaks representing the two sperm populations are shown. The peak at lower intensity corresponds to Y-chromosome-bearing sperm because they have about 4% less DNA than those with the X. Simulated distributions for three proportions of X sperm are shown.

coefficients of variation (CV). The CV was constrained to be the same for the two curves but the other parameters were independently varied. The relative areas of the two peaks gave the respective proportions of X and Y sperm, and the ratio of their means gave their relative DNA content. About 5,000 sperm nuclei were measured for each analysis. Experience with the quality of the fitting procedure indicates that enrichment of either the X or Y sperm population to 55% or greater could be unambiguously detected (Pinkel et al., 1982a,b; Garner et al., 1983).

Discussion

The evidence that the two peaks seen in the flow cytometric measurements represent the two sperm populations is overwhelming. First, we have measured sperm whose X-Y separations are expected to range from zero [the homogametic cockerel (Garner et al., 1983)] to over 9% [the Microtus oregoni (Pinkel et al., 1982a)] based on chromosomal length measurements from karyotypes (Moruzzi, 1979). In all cases the flow cytometric measurements and length-based estimates were in agreement. Second, the separation of the two peaks in mouse sperm increased from 3.2% in normal mice to 4.9% in mice known to be carrying a translocation (Cattanach) of a piece of chromosome 7 to the X chromosome (Pinkel et al., 1982b). This is in agreement with the expectation based on chromosomal length measurements, on DNA content measurements of individual mouse chromosomes (Disteche et al., 1981) and on measurements of spermatids from normal translocation carrying mice (Meistrich et al.,
Finally, flow cytometric-based cell sorting has been used to purify the sperm populations of Microtus oregoni (Pinkel et al., 1982a). Flow cytometric analysis after sorting showed enrichments over 80%. This sorted population is the nearest one can achieve to a positive control because no natural source of sperm has been verified to be enriched in one population.

The semen samples measured in this study were processed with techniques designed to influence the sex ratio of the offspring by physically separating the sperm populations. The flow cytometric technique we used would not be useful for monitoring sex selection procedures that differentially alter the fertility of the sperm populations without separating them. Our results are limited to the samples measured and can not be generalized to all separation techniques of a particular class or to uses of the same technique at other times or by other investigators. This emphasizes the usefulness of a rapid assay such as the one presented here, because analyses must be performed on many samples to guide development of separation techniques and to perform quality controls once one is proven to be effective.

The accuracy of the enrichment determination is governed by the quality of the fit between the measurements and the two Gaussian distributions used by the computer to represent the X and Y peaks. Enough cells are measured so that the statistical errors are negligible. Sometimes the experimental distributions deviate slightly from symmetrical Gaussian shape due to imperfect instrumental operation or difficulties with the staining. In these cases it is apparent that the data would be better fit by a pair of slightly skewed peaks. These small deviations result in uncertainty in the propor-

<table>
<thead>
<tr>
<th>Source</th>
<th>Enrichment technique</th>
<th>Enriched for</th>
<th>X-bearing sperm, %a</th>
<th>No. b</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Convection-counter-streaming-galvanization</td>
<td>X</td>
<td>49.9 ± 1</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Y</td>
<td>50.3 ± .8</td>
<td>9</td>
</tr>
<tr>
<td>B</td>
<td>Albumin gradient</td>
<td>Y</td>
<td>49.3 ± .9</td>
<td>10</td>
</tr>
<tr>
<td>C</td>
<td>Albumin gradient</td>
<td>Y</td>
<td>51</td>
<td>1</td>
</tr>
<tr>
<td>D</td>
<td>Density gradient “Chemical”</td>
<td>X</td>
<td>50.6 ± 4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>X</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>E</td>
<td>Electromotility</td>
<td>X</td>
<td>49.0 ± 2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Y</td>
<td>48.5 ± 2</td>
<td>2</td>
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<td>F</td>
<td>Sex specific antigen</td>
<td>X</td>
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<td>1</td>
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<td></td>
<td></td>
<td>Y</td>
<td>49</td>
<td>1</td>
</tr>
<tr>
<td>G</td>
<td>Sex specific antigen</td>
<td>X</td>
<td>49</td>
<td>1</td>
</tr>
</tbody>
</table>

Values are mean ± SE.

Number of independent samples.

Semen prepared with this technique did not alter the sex ratio in breeding trials (R. H. Foote, personal communication).

Semen was split into control and experimental aliquots and extended with tris-egg yolk without glycerol (Howard et al., 1981). Sperm were isolated by layering 2 ml of extended sperm (10⁸/ml) on a step gradient prepared from bovine serum albumin (BSA) dissolved in tris-egg yolk extender that had been centrifuged (20,000 × g, 30 min.). The gradient consisted of 2 ml 10% BSA under 6 ml 6% BSA in 14 × 100 mm tubes and was held at 37 C. One hour after application of the sperm, the top 2 ml were removed. After an additional 30 min the 6 ml 6% BSA were removed. The sperm that had penetrated to the 10% BSA after the 90-min swim were concentrated and extended in tris-egg yolk. Control semen was cooled to 5 C immediately after extension and treated sperm were cooled to 5 C after concentration and re-extension. Both aliquots were mixed with an equal volume of extender consisting of 7% glycerol, packaged in .5-ml straws and frozen to −196 C (R. P. Amann, personal communication).

Semen prepared with this technique did not alter the sex ratio in breeding trials (Beal et al., 1984).
tions of sperm assigned to the X and Y populations, thus enrichments need to be greater than 55:45 in either direction to be reliably detected. Note that in figure 2 a 60% X population causes a marked change in the shape of the measured fluorescence distribution. This would not be obscured by slight skewing. The accuracy of our analysis is adequate for samples presumed to be enriched sufficiently to be of practical utility (e.g., 80% X-chromosome-bearing sperm).

The flow cytometric technique we have presented can determine the relative proportions of the two sperm populations in a sample, but no determination is made about subsequent fertility. An in vitro fertilization assay that allows determination of chromosomal complement of individual sperm (Rudak et al., 1978; Martin et al., 1983; Brandriff et al., 1984) might be useful for samples in which the fertility of the two sperm populations was differentially altered. However, because much current sex preselection research (table 1) centers on sperm separation, the flow cytometric system described here offers the first practical and widely applicable means of rapidly screening samples of “sexed” semen.

Literature Cited


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