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Sex Preselection in Rabbits: Live Births from X and Y Sperm Separated by DNA and Cell Sorting

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ABSTRACT

Intact, viable X and Y chromosome-bearing sperm populations of the rabbit were separated according to DNA content with a flow cytometer/cell sorter. Reanalysis for DNA of an aliquot from each sorted population showed purities of 86% for X-bearing sperm and 81% for Y-bearing sperm populations. Sorted sperm were surgically inseminated into the uterus of rabbits. From does inseminated with sorted X-bearing sperm, 94% of the offspring born were females. From does inseminated with sorted Y-bearing sperm from the same ejaculates, 81% of the offspring were males. The probability of the phenotypic sex ratios differing from 50:50 were $p<0.0003$ for X-sorted sperm and $p<0.004$ for Y-sorted sperm. Thus, the phenotypic sex ratio at birth was accurately predicted from the flow-cytometrically measured proportion of X- and Y-bearing sperm used for insemination.

INTRODUCTION

The ability to preselect the sex of offspring of agriculturally important animals would have a significant impact on the genetics and economics of livestock production. The only established difference between X and Y chromosome-bearing sperm is the quantity of DNA in the sex chromosome. This difference can be measured in individual sperm using flow cytometric analysis (Pinkel et al., 1982b; Garner et al., 1983; Johnson and Pinkel, 1986; Johnson et al., 1987a). Numerous physical, biochemical, and immunological methods of semen treatment have been proposed for altering the sex ratio of offspring (for a recent review, see Amann, 1989). No conclusive proof of X- and Y-bearing sperm enrichment based on subsequent fertility has been demonstrated. Further, semen processed by most of the proposed physical, biochemical, or immunological methods has been tested by DNA analysis in recent years (Pinkel et al., 1985; Johnson, 1988a) without evidence of sex ratio alteration.

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Flow cytometric measurement of sperm DNA has been used to determine the ratio of X and Y chromosome-bearing sperm in animal semen (Johnson and Pinkel, 1986; Johnson et al., 1987a). Flow-sorting was used to separate the X- and Y-bearing sperm of the vole (Pinkel et al., 1982a) and chinchilla (Johnson et al., 1987b). However, in these studies, preparation procedures damaged the viability of DNA. Recently, bull, boar, and ram sperm nuclei were flow-sorted into separate X and Y sperm populations with greater than 90% enrichment for X- or Y-bearing sperm, and the sorted sperm nuclei were shown to be capable of pronuclear development after microinjection into hamster eggs (Johnson and Clarke, 1988).

In this report, intact, viable rabbit sperm were flow-cytometrically separated into X and Y populations on the basis of relative DNA content. The separation of X- and Y-bearing sperm was verified by determining the sex ratio of offspring born.

MATERIALS AND METHODS

Semen Preparation and Flow Sorting

Semen was collected from two mixed breed mature bucks (12 ejaculates) by use of an artificial vagina and

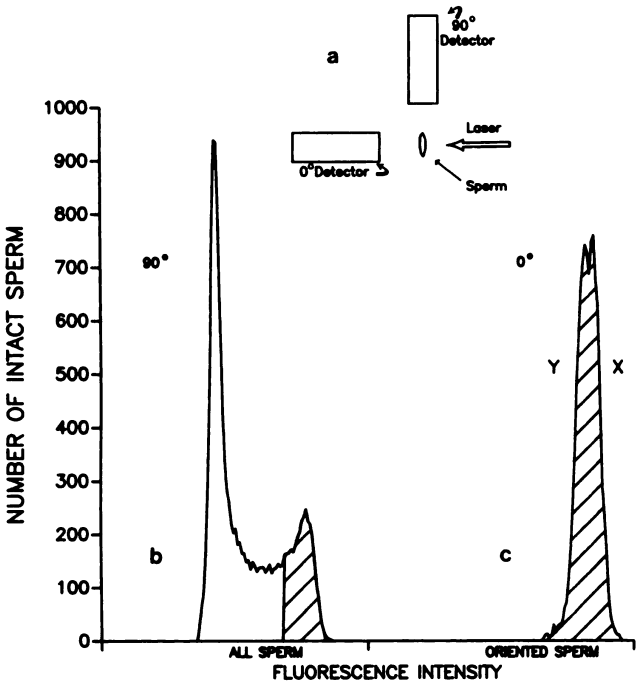

FIG. 1. *a*) Top view of the optical arrangement of the EPICS V Flow Cytometer/Cell Sorter (Coulter Corporation, Hialeah, FL) modified for sperm. Sample flow is perpendicular to the plane of the page. Fluorescent detectors measure fluorescence emitted in cones centered 0 and 90 degrees to the direction of the propagation of the laser beam. *b*) Histogram from the 90-degree detector showing the gating window (*hatched area*; 17 channels wide), selecting those sperm with their edges toward the 90-degree detector (properly oriented), and their flat faces toward the propagation of the laser beam and the 0-degree detector. *c*) Histogram illustrating the output from the signals captured by the 0-degree detector from the sperm falling within the gate window of the 90-degree detector.

a teaser female. Sperm concentration was determined with a hemocytometer. The semen was diluted with Tris buffer (tris(hydroxymethyl)aminomethane, 2.52 g; D glucose, 1.04 g; citric acid, 1.28 g), pH 6.9, to a concentration of 10×10^6 sperm per ml. Bisbenzimide H33342 fluorochrome (Calbiochem-Behring, La Jolla, CA) was added to achieve a final concentration of 9 μM. The samples were incubated for 1 h at 35°C to aid penetration of the fluorochrome. Intact sperm were sorted on an EPICS V flow cytometer/cell sorter (Coulter Corporation, Hialeah, FL) modified for sperm (Johnson and Pinkel, 1986; Johnson, 1988b; Fig. 1). The stained intact sperm were excited in the ultraviolet (UV; 351 and 364 nm) lines of a 5-watt 90-5 Innova Argon-ion laser (Coherent, Inc., Palo Alto, CA) operating at 200 mW. A 76-μm jet-in-air flow tip was used. Data were collected as 256-channel histograms. Sheath fluid was 10 mM phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Sperm were sorted into Test buffer (Graham et al., 1972) modified by adding 0.5% Equex STM (Nova Chemical, Inc., Scituate, MA) and 20% hen's egg yolk. Microfuge tubes (0.5 ml) served as receptacles for the sorted sperm.

To sort X- and Y-bearing intact sperm, electronic sorting windows were set on the fluorescent signal peaks obtained from the 0-degree detector measurement of the intact sperm sample (Fig. 1). Sorting windows were 10 channels wide for each of the Y and X peaks, respectively. Sorted sperm were concentrated by incubating at room temperature for 1 h, after which the more dilute upper fraction was discarded. The concentrated fraction was used for insemination 1–4 h later.

Surgical Insemination

Mature New Zealand White does were injected with 150 IU human chorionic gonadotropin (hCG) to induce ovulation, which was expected to occur 10 h later. Seven hours after treatment with hCG, the does were injected with Ketamine hydrochloride containing acepromazine, and anesthesia was maintained under halothane and oxygen. The uterus was exposed by midline incision, and 100 μl of sorted, unsorted, or recombined sperm (approximately 3×10^5) was placed into the lumen of the anterior end of each uterine horn through a 21-gauge needle. The phenotypic sex of the offspring was determined shortly after birth and was reconfirmed at various times up to 10 wk of age.

Reanalysis of Sperm for DNA Content

The relative DNA content of the sorted intact sperm used for insemination was determined by flow cytometric analysis of sperm nuclei from each of the respective fractions. Sperm nuclei were prepared from an aliquot of intact sorted sperm by sonication for 10 s in PBS and centrifuged. The sperm pellet was resuspended in PBS and Bisbenzimide H 33342 to a concentration of 9 μm (Johnson et al., 1987b). The samples of sperm nuclei were analyzed under the same instrument conditions as described above, with the exception that they were not sorted. The proportion of X- and Y-bearing sperm in nuclei samples or in sorted sperm samples was determined by fitting a pair of Gaussian distributions to the histograms from the 0-degree detector (Johnson et al., 1987b).

TABLE 1. Predicted and actual sex ratios of offspring after intrauterine insemination of sorted X and Y chromosome-bearing rabbit sperm.

Treatment of sperm	Number of does		Total no. of young born	Percentage and number of offspring			
				Predicted[a]		Actual[b]	
	Inseminated	Kindled		% Males	% Females	% Males (N)	% Females (N)
Sorted Y	16	5	21	81	19	81 (17)	19 (4)
Sorted X	14	3	16	14	86	6 (1)	94 (15)
Recombined X and Y	17	5	14	50	50	43 (6)	57 (8)
Total	47	13	51	---	---	47 (24)	53 (27)

[a]Represents the results of reanalysis for relative DNA content of aliquots of sorted X- and Y-bearing sperm populations.

[b]Represents actual births.

The data were analyzed statistically by a binomial distribution analysis (SAS Institute). The 95% confidence limits were also calculated (Natrella, 1966).

RESULTS

In an initial experiment, the fertilizing capacity of sperm was not seriously affected by the sorting process. Each of 39 ova were fertilized when recovered 40 h post-insemination from 5 does inseminated with stained, sorted sperm. All 7 ova from another doe were unfertilized. Nine ova, all fertilized, were recovered from 2 does inseminated with unstained, unsorted sperm. All fertilized ova were 8–16 cells.

The ratio of X to Y sperm in the sorted sperm fractions used for insemination accurately predicted the phenotypic sex ratio of the offspring (Table 1). Reanalysis of an aliquot of the sorted Y-bearing sperm population used for insemination indicated that 81% of the sperm were Y-bearing. The offspring from these inseminations were 81% male. Reanalysis of an aliquot of the sorted X-bearing sperm population used for insemination indicated that 86% of the sperm were X-bearing, and the offspring were 94% female. Does were also inseminated with sorted X- and Y-bearing sperm populations that were recombined immediately before insemination. From the concentration and purity of the sorted fractions, we assumed that approximately equal numbers of X- and Y-bearing sperm were present in the recombined samples. The phenotypic sex resulting from these inseminations was 57% female and 43% male (Table 1). The numbers in Table 1 include 8 young that died between 2 and 4 days of age. Those young that died were spread evenly across the three groups (3 females in sorted X, 3 males in sorted Y, and 2 females in recombined X and Y; Table 1). No phenotypic abnormalities were found. All other offspring have grown to adulthood without complication.

The probability of the observed phenotypic sex ratio results (Table 1) differing from 50:50 was $p<0.0003$ for does inseminated with X-sorted sperm and $p<0.004$ for does inseminated with Y-sorted sperm. The proportion of male and female offspring resulting from the insemination of recombined X and Y sperm did not differ from 50:50 ($p = 0.40$). These probabilities agree with those established to test against a 50:50 sex ratio for samples of similar size (Moore and Gledhill, 1988).

Figure 2 illustrates the typical analysis for relative DNA content for sperm nuclei, for intact sperm, and for sperm nuclei prepared from sorted intact sperm. The difference in DNA content between rabbit X- and Y-bearing sperm is only 3%, hence the overlap in the original histogram (Fig. 2b). The shoulders on the peaks in Figures 2c and 2d show the approximate amounts of impurity in each fraction.

Figure 3 illustrates the close relationship between the predicted percentage of male offspring and the actual offspring born. The bar for predicted percentage of males from X-sorted sperm represents a mean of 10 reanalyses of sorted sperm (14 ± 0.77%, SEM) and mean percentage of males from Y-sorted sperm (81 ± 1.90%, SEM). The 95% confidence interval (one-sided) for the proportion of females resulting from the insemination of the sorted X fraction is $0.746 \leq p \leq 1.0$, and for the proportion of males from insemination of the sorted Y fraction, $0.616 \leq p \leq 1.0$. The 95% confidence interval (two-sided) for the proportion of females when recombined X and Y sperm fractions were inseminated was $0.312 \leq p \leq 0.794$, and for males, $0.206 \leq p \leq 0.688$.

DISCUSSION

Accurate measurement of mammalian sperm DNA content using flow cytometry and cell sorting is difficult because the sperm nucleus is highly condensed and

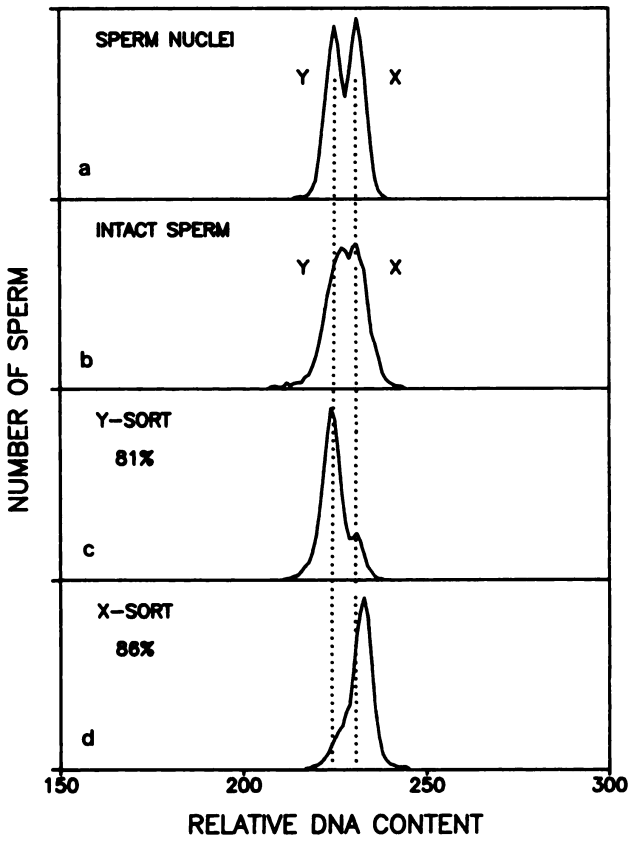

FIG. 2. Illustrates typical histogram output from the 0-degree detector for analysis and sorting of rabbit sperm. *a*) A typical frequency distribution from analysis of rabbit sperm nuclei; the calculated difference in DNA between the X and Y peaks is 3.0%. *b*) The distribution that can be obtained from intact viable sperm that are being sorted. *c*) Represents the frequency distribution obtained from reanalysis of sperm nuclei from an aliquot of sorted Y-bearing intact sperm. *d*) Represents the frequency distribution obtained from reanalysis of sperm nuclei prepared from an aliquot of sorted X-bearing, intact sperm. The *vertical dotted line* illustrates the actual relationship, in terms of relative DNA content, of the various analyses, which were all run under the same instrument conditions.

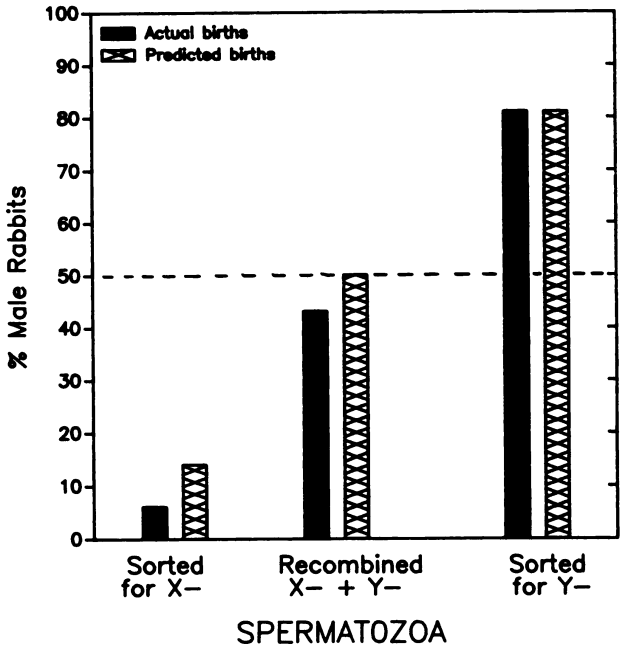

FIG. 3. Bar graph illustrating the similarity of the predicted percentages of male offspring by measuring DNA of X and Y sperm to the sex of actual offspring born.

flat in shape, which makes stoichiometric staining difficult and causes stained nuclei to have a high index of refraction. These factors contribute to emission of fluorescence preferentially from the edge or thin plane of the sperm nucleus. In most flow cytometers and sorters, the direction of sample flow is orthogonal to the direction of propagation of the laser beam and the optical axes of the fluorescence detection. Consequently, fluorescence measurement is most accurate when the sperm fluorescence is excited and measured on an axis perpendicular to the plane of the sperm head (Pinkel et al., 1982b). At relatively low sample flow rates, hydrodynamics are used to orient tailless sperm so that DNA content can be measured precisely on 60–80% of the sperm (50–150 sperm/s) passing in front of the laser beam of the modified EPICS V flow cytometer (Johnson and Pinkel, 1986).

Intact sperm (with tails), however, whether viable or nonviable, cannot be oriented as effectively as tailless sperm nuclei (Johnson, 1988b). However, the 90-degree detector can still be used to select the population of properly oriented intact sperm (Fig. 1) to be measured by the 0-degree detector. Since no hydrodynamic orientation is attempted, the sample flow rate can be much higher, which compensates somewhat for the fact that only 15–20% of intact sperm pass through the laser beam with proper orientation (Fig. 1). In this study, the overall flow rate was approximately 2500 intact sperm/s. The intact X- and Y-bearing sperm fractions were sorted simultaneously from the population of input sperm at a rate of 80–90 X-bearing sperm/s and 80–90 Y-bearing sperm/s (Fig. 2).

Natural mating generally results in a kindling rate of 80% and a litter size of 6 young born (Casady et al., 1966). The lower kindling rate (28%) and litter size (3.9) in this study are thought to be due to reduced egg pickup because of manipulation of the uterus during

surgical insemination and the possible effects of the DNA-bound fluorochrome (unpublished data). The stage of gestation at which embryonic death may occur has not been established.

Earlier work in this laboratory has shown chromosome breakage in the developing sperm pronucleus after vole sperm nuclei were flow-sorted and microinjected into hamster eggs (Libbus et al., 1987). Those sperm, however, had been sonicated, stained, sorted and microinjected, whereas in this study the sperm were stained and sorted only. Other attempts have also been made to flow-sort sperm for insemination. These attempts have resulted in very low pregnancy rates (Morrell et al., 1988).

The results described here represent a significant advance toward the goal of sex preselection for mammals. However, several factors mitigate against widespread application of this methodology at the present time: (1) The limitation on number of sperm that can be sorted in a reasonable period of time (about 3.5×10^5 per hour) eliminates use of this procedure for producing sexed semen for standard cervical artificial insemination in most mammals. (2) The increased embryo mortality presumed to be related to the presence of the fluorochrome on the DNA. (3) The cost of the modified flow cytometer/sperm-sorting instrumentation (approximately $250,000). However, none of these factors appear to represent insurmountable difficulties. In fact, the current procedure might be effectively used in conjunction with in vitro fertilization, especially with respect to cattle.

These data provide conclusive evidence that flow-cytometrically determined sperm DNA content can be used as a differentiating marker between X- and Y-bearing sperm for purposes of determining sperm sex ratio, that measurement of DNA in separated X- and Y-bearing sperm populations can be used to predetermine the sex of offspring, and that flow-sorting is effective for separating viable X- and Y-bearing sperm populations suitable for production of offspring.

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