MOLECULAR REPRODUCTION AND DEVELOPMENT 50:323–327 (1998)

Comparative Motility of X and Y Chromosome–Bearing Bovine Sperm Separated on the Basis of DNA Content by Flow Sorting

L.M. PENFOLD,[1] C. HOLT,[2] W.V. HOLT,[2] G.R. WELCH,[1] D.G. CRAN,[3] AND L.A. JOHNSON[1]*

[1]*Germplasm and Gamete Physiology Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland*
[2]*Institute of Zoology, Regents Park, London, England*
[3]*Mastercalf, Ltd., Aberdeen, Scotland*

ABSTRACT A combination of flow cytometric sperm sorting of X and Y chromosome–bearing sperm (X and Y sperm) and computer-assisted sperm analysis (CASA) for measuring sperm motility allows assessment of motion parameters in the two populations. Bull sperm were separated into X and Y populations by flow cytometry following staining with the DNA-binding dye Hoechst 33342. The motion parameters differed depending on sperm concentration. Decreasing sperm concentration resulted in higher velocities and straighter trajectories. The concentrations of control (stained-unsorted and unstained-unsorted) and flow-sorted sperm were therefore adjusted to similar numbers (5×10^6 sperm per milliliter). Samples of sorted X and Y sperm and control sperm were transferred to prewarmed slides on a heated stage (37°C) and their motion video recorded for 2 min using a magnification of ×100 and a high-resolution camera. The sperm analysis was carried out on a Hobson Sperm Tracker (HST) using HST 7 software. The following motion parameters were measured: curvilinear, straight-line, and average path velocity; mean angular displacement (MAD); beat cross-frequency; amplitude of lateral head displacement; linearity (LIN); and straightness of path (STR). Sperm movement was unaffected by staining with Hoechst 33342, excitation by ultraviolet (UV) light, or the physical process of cell sorting. Significant differences were seen between X and Y sperm for MAD, LIN, and STR. No difference was observed for the other parameters. The results indicate that in a simple salts solution, Y bull sperm do not swim faster than X sperm but may be distinguished from X sperm on the basis of LIN and STR. *Mol. Reprod. Dev. 50:323–327, 1998.*

Key Words: CASA; sperm; flow cytometry; X and Y chromosome–bearing sperm

INTRODUCTION

Gender preselection has been a desire of humankind for generations. Numerous research studies have been conducted to achieve this goal for livestock production as well as for the human population (Amann, 1989). Among the methods devised to separate X and Y sperm by apparent "physical characteristics" is the albumin gradient method described by Ericsson et al. in 1973 and applied by various clinics in human medicine for the past 15 years (Beernink et al., 1993). Supposedly, the faster swimming speed of the smaller Y sperm enables those sperm to reach the bottom of the gradient before the X-bearing sperm. No conclusive proof has ever been put forth that this view is indeed true, since no method previously existed by which sperm could be separated into nearly pure X and Y sperm populations (Johnson, 1992). This is so despite the fact that the albumin method has been offered to clinicians for many years as an effective method for preselecting sex.

The X chromosome is larger than the Y chromosome and therefore contains more DNA. It might be expected that differences in DNA mass between X and Y chromosome–bearing sperm (Johnson et al., 1989) would influence swimming velocity. However, it also might be expected that any difference in motion parameters between X and Y sperm would be subtle and that experimental conditions to date may not have been sufficiently sensitive to determine these differences. With the development of a validated sperm sexing method (Johnson, 1991; Johnson et al., 1989, 1993) based on the only known difference in X and Y chromosome sperm (DNA content) and the ability of computer-assisted sperm analysis (CASA) to measure motility in a semiquantitative manner, it became possible to test the hypothesis. CASA has been used extensively in the past 10 years with increasing usefulness for assessing sperm quality (Holt, 1996).

Separation of X and Y sperm based on a difference in DNA content has been validated by the birth of offspring for the rabbit (Johnson et al., 1989), boar (Johnson, 1991), bull (Cran et al., 1993), and sheep

L.M. Penfold's present address: White Oak Conservation Center, Animal Science, Yulee, FL.

*Correspondence to: Dr. L.A. Johnson, Germplasm and Gamete Physiology Laboratory, Agricultural Research Service, U.S. Department of Agriculture, BARC-East, B-200, Beltsville, MD 20705.

Received 3 October 1997; Accepted 20 January 1998

(Cran et al., 1997); it also has been demonstrated that X and Y sperm can be sorted into separate populations based on DNA in the human (Johnson et al., 1993). In general, separated populations have been determined to be 80–90% pure for populations of X or Y chromosome–bearing sperm. The CASA system used in this study, the Hobson Sperm Tracker (HST), is able to carry out continual assessment of sperm in real time, allowing a new approach to sperm assessment. The HST has been thoroughly investigated as an effective CASA system with high precision and coefficients of variation below 3% for each motion parameter measured (Holt, 1996). In contrast to other CASA systems that track an individual sperm for approximately 30 frames, the HST can track a sperm for as long as it remains in the area being viewed. Increasing the number of frames over which a sperm is tracked increases the accuracy of the data (Owen and Katz, 1993).

The objective of this study was to evaluate the motility of bull sperm (a factor of swimming speed) that had been sorted flow cytometrically into separate X and Y chromosome–bearing sperm populations. Sperm were prepared for flow sorting on the basis of a difference in DNA mass, and the sorted collected sperm were measured for the various parameters of motility that can be assessed with CASA.

MATERIALS AND METHODS

Semen Collection and Preparation

Ejaculates from three bulls were collected three times over 9 days in Experiment 1, and ejaculates from two bulls were collected four times over 8 days in Experiment 2. The semen was diluted in HEPES-buffered medium (130 mM NaCl, 4 mM KCl, 14 mM fructose, 1 mM $CaCl_2$, 0.05 mM $MgCl_2$, and 1 mM HEPES) containing 0.1% bovine serum albumin (HEPES-BSA) (Johnson et al., 1989) to a concentration of 15×10^6 sperm per milliliter. Samples of diluted semen were prepared as follows: stained with 5 µg/ml of Hoechst 33342 fluorochrome (HO42; Calbiochem-Behring Corp., La Jolla, CA) and incubated for 35 min at 35°C (Johnson et al., 1989) and incubated for 35 min 35°C in the absence of HO42 stain (control; hereafter referred to as *unstained*). After initial incubation, propidium iodide (PI) was added to all samples to give a concentration of 25 µg/ml, and the samples were further incubated for 5 min at 30°C in order to stain the dead sperm in each population to increase sorting efficiency (Johnson et al., 1994).

Flow Cytometric Sorting of Sperm

Stained sperm were sorted with a modified Epics V flow cytometer/cell sorter (Coulter Corporation, Miami, FL) modified for the analysis of X and Y chromosome–bearing sperm based on DNA content (Johnson and Pinkel, 1986). The fluorochrome-stained sperm were excited in the ultraviolet wavelength (351, 364 nm) of a 5-W 90-5 Argon Inova laser (Coherent, Inc., Palo Alto, CA) operating at 175 mW, and fluorescence was detected through 418-nm long-pass filters. A 76-µm jet-in-airflow tip was used. Data were collected as 256-channel histograms. Sheath fluid was 0.1 M phosphate-buffered saline (PBS) containing 0.1% BSA. All viable sperm sorting (USDA Beltsville Sperm Sexing Technology) was carried out at room temperature (~22°C), as described by Johnson et al. (1989).

Reanalysis of Sorted X and Y Chromosome–Bearing Sperm

Sorted sperm were collected during the sampling process into 500-µl presiliconized microfuge tubes. After completion of the video recording of X- and Y-sorted sperm, the remainder of the sorted sample was sonicated for 10 sec to remove the sperm tails. Additional (0.5 µg/ml) HO42 was added to the sample before incubating at 35°C for 15 min (Johnson et al., 1987). Samples were then reanalyzed at room temperature on the modified flow cytometer for DNA content to determine the purity of the collected sperm.

Computer-Assisted Analysis of Sperm Movement

Aliquots of 7 µl from each sperm concentration in Experiment 1 and each treatment group in Experiment 2 (X-sorted sperm, Y-sorted sperm, stained-unsorted control, and unstained control) were transferred to alcohol-washed, prewarmed (37°C) standard microscope slides. The aliquots were covered with 18 × 18 mm coverslips and immediately transferred to the warm stage (37°C) of a Zeiss Axiophot microscope (Carl Zeiss, Thornwood, NY). The microscope was equipped with a ×10 phase objective and a high-resolution video camera (Hamamatsu CCD Model) connected to a C2400 camera control unit (Hamamatsu CCD). The slide was left for 30 sec on the stage for the sample to equilibrate and minimize drift. Sperm motion was continuously recorded for 2 min, and care was taken to ensure similar light levels for each sample. Motion parameters (Boyer et al., 1989) were examined using HST with HST version 7 software and a frame rate of 25 Hz (Hobson Tracking Systems, Ltd., Sheffield, UK). Definitions of each motion parameter are as follows:

Curvilinear velocity (VCL): velocity over the total distance moved, i.e., including all deviations of sperm head movement.

Straight-line velocity (VSL): velocity calculated using the straight-line distance between the beginning and end of the sperm track.

Average path velocity (VAP): velocity over a calculated, smoothed path, i.e., a shorter distance than that used for calculating VCL.

Mean angular displacement (MAD): the mean angular displacement of the sperm head around the curvilinear path.

Beat cross-frequency (BCF): the frequency with which the actual track crosses the smoothed track.

Amplitude of lateral head displacement (ALH): the average value of the extreme side-to-side movement of the sperm head in each beat cycle.

TABLE 1. Means of Velocity Head Movement and Trajectory Variables for Sperm Concentrations of 1, 5, and 10 × 10^6 sperm/ml

Sperm ×10^6/ml (n = 9)	No. of sperm counted	Motion parameter (μm/sec)							
		VCL	VSL	VAP	MAD	BCF	ALH	LN	STR
SE ±		9	10	11	2.1	0.3	1.4	4	5
1	645	195[a]	99[a]	138[a]	86[a]	5[a]	13[a]	48[a]	74[a]
5	1793	204[a]	98[a]	143[a]	85[a]	5[a]	14[a]	47[a]	76[a]
10	3364	191[a]	70[b]	110[b]	85[a]	6[a]	13[a]	35[b]	67[b]

[a,b]Column means followed by different superscripts are significantly different ($P \geq 0.05$).

Linearity (LIN): ratio of distances (as a percentage) of straight-line track length to actual track length (this value is 100% for a completely linear track).

Straightness of path (STR): straight-line velocity divided by the average path velocity.

Conditions for HST Setup

Filter settings were 1,1,1 and 2 for 1, 2, 3, and 4, respectively. Predict was off and search radius was 33.2 μm. Minimum track points were 15 frames. Contrast thresholds were +12/−8. Tracking time was 2 min.

Experiment 1: Effect of Sperm Concentration on Motility

Since the process of flow cytometry and cell sorting dilutes sperm samples from their initial concentration, the effects of concentration only on motion parameters were examined initially. Bull semen (n = 9 total ejaculates) was diluted in HEPES-BSA to concentrations of 1, 5, and 10 × 10^6 sperm per milliliter and incubated at 37°C for the duration of the motility analysis. Three aliquotes from each concentration were video recorded for CASA in a 3 × 3 Latin square design.

Experiment 2: Motility of X and Y Chromosome–Bearing Sperm

Bull semen (n = 8 total ejaculates) was diluted in HEPES-BSA and stained with HO42 as described above. Stained samples were flow sorted into X and Y chromosome–bearing sperm according to established protocols (Johnson et al., 1989). Sperm were sorted directly from the modified cell sorter into 500 μl of Test-yolk (20% v/v) buffer (Johnson et al., 1989) before sorting. Control samples of stained and unstained sperm that were not subjected to sperm sorting were diluted 1:15 with PBS containing 0.1% BSA to mimic the effects of dilution that occur during routine flow sorting of sperm. After dilution, 400 liters of each of the control samples was transferred to 500-μl presiliconized microfuge tubes that had been rinsed with HEPES–1% BSA (~22°C) also containing 50 μl of Test-yolk buffer at room temperature (~24°C). After addition to the Test-yolk, control stained and unstained samples were kept at room temperature until approximately 300,000 (400 μl) sorted sperm had been collected to comprise an X and Y chromosome–bearing sperm population (~1 hr). From the results of Experiment 1, an effect of concentration on motility was observed for some motion parameters. Therefore, samples in Experiment 2 were adjusted to a standard concentration before motility was video recorded. Following collection of sorted samples, all tubes, including controls, were centrifuged at 350*g* for 4 min. Each pellet was resuspended in 66 μl of HEPES-BSA to a concentration of approximately 5 × 10^6/ml. The resuspended sperm were transferred to a heat block at 37°C for the duration of the video recording (45 min). Four aliquots from each treatment group were video recorded for CASA in a 4 × 4 Latin square design to eliminate time effects.

Statistical Analysis

Weighted means were taken into account for differences in the numbers of sperm analyzed. A mixed-model ANOVA (SAS, 1994) was used to avoid underestimating the variance of the group means and where bull and day were considered random effects. Differences in motility parameter means for X and Y sperm populations were compared using the least significant difference test.

RESULTS

Reanalysis of Separated X and Y Chromosome–Bearing Sperm

Aliquots of the sorted X and Y chromosome–bearing sperm were reanalyzed for DNA content. Flow cytometric analysis confirmed purities of ≥85% for X and Y sperm, respectively.

Experiment 1: Effect of Sperm Concentration on Motility

No differences in motion parameters were seen between concentrations of 1 × 10^6 and 5 × 10^6 sperm per milliliter (Table 1; P = 0.05). Lower velocities (VSL, VAP) and lower measurements of trajectory (LIN, P = 0.01; STR, P = 0.05) were recorded for concentrations of sperm at 10 × 10^6 sperm per milliliter compared with 1 × 10^6 and 5 × 10^6 sperm per milliliter (see Table 1).

Experiment 2: Motility of X and Y Chromosome–Bearing Sperm

For clarity of presentation, data are presented in two tables. The staining of sperm with HO42 had an effect of velocity in VSL and MAD in stained samples, but excitation of the stain with ultraviolet light and the

TABLE 2. Means of Velocity and Head Movement Variables for Unstained, Hoechst Stained-Unsorted, and X and Y Separated Sperm

Treatment	No. of sperm counted	Motion parameter (μm/sec) VCL	VSL	VAP	MAD	BCF	ALH
SE ±		8	8	9	0.7	0.1	0.6
Unstained	3713	257[a]	86[a]	118[a]	85[a]	6.5[a]	16[a]
Stained, unsorted	4145	268[b]	91[a]	123[a]	87[b]	6.6[a]	16[a]
X sorted	3191	263[a]	84[a]	118[a]	87[b]	6.8[a]	16[a]
Y sorted	4116	256[a]	72[a]	105[a]	85[a]	6.8[a]	16[a]

[a,b]Column means followed by different letters are significantly different from each other at the $P = 0.05$ level.

TABLE 3. Means of Trajectory Variables for Unstained, Hoechst Stained-Unsorted, and X- and Y-Sorted Sperm

Treatment	No. of sperm counted	Motion parameter (μm/sec) LIN	STR
SE ±		3	5
Unstained	3713	31[a]	64[a]
Stained, unsorted	4145	32[a]	65[a]
X sorted	3191	31[a]	65[a]
Y sorted	4116	27[b]	58[b]

[a,b]Column means followed by different letters are significantly different from each other at the $P = 0.05$ level.

physical process of flow sorting appeared to have little or no effect on motility in stained X- and Y-sorted sperm when compared with unstained-unsorted sperm (Table 2; $P = 0.05$). Sperm sorted for the Y chromosome were not found to have higher velocities when compared with X sperm (see Table 2; $P \leq 0.05$). Significant differences between LIN ($P = 0.04$) and STR ($P = 0.01$) were seen between X and Y sperm (Table 3). Although MAD also was significantly different between X and Y sperm (see Table 2; $P = 0.04$), it was not significantly different between Y and unstained-unsorted controls (see Table 2; $P = 0.9$).

DISCUSSION

The results of this study do not support the frequently held view that Y chromosome–bearing sperm swim faster than X chromosome–bearing sperm ($P \leq 0.05$). Sperm velocities (VCL, VSL, or VAP) were not statistically different between X and Y chromosome–bearing sperm. However, significant differences were seen for LIN ($P = 0.04$) and STR ($P = 0.01$) between X- and Y-sorted sperm. Although MAD was found to be significantly different between X and Y sperm, this result was unexpected, since MAD is normally highly conserved among sperm populations (W Holt, personal communication).

The effect of sperm concentration on velocity, previously described for ram and human sperm (Yi Lui et al., 1991; Suttiyotin and Thwaites, 1992), also was confirmed for bull sperm in this study. From the results in Experiment 1, the importance of standardization of sample concentration between control and flow-sorted samples was confirmed. Samples collected after flow sorting have a low concentration ($<1 \times 10^6$ sperm per milliliter); thus concentrations of all samples were adjusted to 5×10^6 sperm per milliliter in this study to standardize video recording of sperm. Higher VCL was seen in sperm samples from Experiment 2. Sperm samples in Experiment 2, including controls, were diluted as a process of or to mimic flow cytometry/cell sorting, and samples were recorded 1 hr after flow sorting or dilution of control samples. The increase in velocity may have been indicative of the onset of the capacitation process, with sperm exhibiting some hyperactivated motility (Suarez et al., 1991) and thus increased velocities, although no increase in velocity or hyperactivated motility was observed visually.

In both experiments, the results for VCL are much greater than those of VSL. This is in contrast to velocities for ram and boar (Holt, 1995) sperm, which have more similar velocities. In this study, values of 13–16 μm were measured for ALH. This magnitude of head movement was further verified by subjective assessment of video-recorded sperm revealing wide side-to-side head movement, while at the same time moving forward linearly, which would account for these discrepancies in velocity.

In this study, CASA was carried out on sperm samples that had been diluted in a buffered medium. In the female reproductive tract, the fluids encountered by the sperm would be significantly more viscous. This increased viscosity would also be true of the albumin procedure for human sperm published by Ericsson et al., 1993. Elevated fluid viscosity has been shown to affect the motility of the sperm (Suarez et al., 1991). It is possible that differences in motility between X and Y sperm may be more or less pronounced in a more viscous medium. Differences in swimming patterns have been observed in Ficoll and cumulus matrix, possibly due to macromolecular differences in structure (Suarez et al., 1991). Interestingly, serum albumin in medium has been shown to have a "shear-thinning" effect, reducing the effects of viscosity as the shear rate increases and altering the efficiency at which a sperm swims (Suarez et al., 1991).

Under the conditions of this study, we conclude that bull Y sperm do not swim faster than bull X sperm. Instead, the difference between X and Y sperm with regard to LIN and STR, which are highly correlated with velocity, indicate that bull X sperm may even swim faster than bull Y sperm. If this were the case, then the

overall mass of the sperm is not exerting a significant affect on motility. It would be interesting to consider whether structures such as the flagellum or other sperm factors are modified after the second meiotic division in a way that could influence sperm motility. Future development of a system that enabled analysis of flagellar movement together with head movements may provide more information on differences in motion parameters between X and Y chromosome–bearing sperm. The findings of this study continue to leave open the possibility that the difference in motility of X and Y chromosome–bearing sperm may be related to the presence of the Y or X chromosome; however, it is doubtful that any subtle difference would be useful for separating X and Y chromosome–bearing sperm.

ACKNOWLEDGMENTS

We are grateful to W. Potts, University of Maryland, for assistance with the statistical analyses. Special thanks to D. Nace for technical assistance with X and Y sperm separation and to P. Graninger for collection of the bull semen.

REFERENCES

Amann RP (1989): Treatment of sperm to predetermine sex. Theriogenology 31:49–60.

Beernik FJ, Dmowski WP, Ericsson RJ (1993): Sex preselection through albumin separation of sperm. Fertil Steril 59:382–386.

Boyer SP, Davis R, Katz DF (1989): Automated semen analysis. Curr Probl Obstet Gynecol 12:172–200.

Brandriff BF, Gordon LA, Haendal S, Singer S, Moore DH, Gledhill, BL (1986): Sex chromosome ratios determined by karyotype analysis in albumin isolated human sperm. Fert Steril 46:678–685.

Budworth PR, Amann RP, Chapman PL (1988): Relationships between computerized measurements of motion of frozen-thawed bull spermatozoa and fertility. J Androl 9:41–54.

Cran DG, Johnson LA, Miller NGA, Cochrane D, Polge C (1993): Production of calves following separation of X and Y chromosome bearing sperm and in vitro fertilization. Vet Rec 132:40–41.

Cran DG, McKelvey WAC, King ME, Dolman DF, McEvoy TG, Broadbent PJ, Robinson JJ (1997): Production of lambs by low dose intrauterine insemination with flow cytometrically sorted and unsorted semen. Theriogenology 47:267.

Davis RO, Katz DF (1992): Standardization and comparability of CASA instruments. J Androl 13:81–86.

Davis RO, Katz DF (1993): Operational standards for CASA instruments. J Androl 14:385–394.

Ellington J, Scarlett J, Meyers-Wallern V, Mohammed HO, Surman V (1993): Computer-assisted sperm analysis of canine spermatozoa motility measurements. Theriogenology 40:725–731.

Ericsson RJ, Langevin CN, Nashimo M (1973): Isolation of fractions rich in human Y sperm. Nature 253:421–424.

Holt C (1995): The use of CASA in predicting boar fertility. Ph.D. thesis, University College, London.

Holt WV (1996): Can we predict fertility rates? Making sense of sperm motility. Reprod Domest Anim 31(suppl 1):17–24.

Jasko DJ, Little TV, Smith K, Lein DH, Foote RH (1988): Objective analysis of stallion semen motility. Theriogenology 30:1159–1167.

Johnson LA (1991): Sex preselection in swine: Altered sex ratios in offspring following surgical insemination of flow sorted X and Y chromosome bearing sperm. Reprod Domest Anim 26:309–314.

Johnson LA (1992): Gender preselection in domestic animals using flow cytometrically sorted sperm. J Anim Sci 70(suppl 1):8–18.

Johnson LA, Pinkel D (1986): Modification of a laser-based flow cytometer for high resolution DNA analysis of mammalian spermatozoa. Cytometry 7:268–273.

Johnson LA, Flook JP, Look MV, Pinkel D (1987): Flow sorting of X and Y chromosome–bearing spermatozoa into two populations. Gamete Res 16:1–9.

Johnson LA, Flook JP, Hawk HW (1989): Sex preselection in rabbits: Live births from X and Y sperm separated by DNA and cell sorting. Biol Reprod 41:199–205.

Johnson LA, Welch GR, Keyvanfar K, Dorfman A, Fugger F, Schulman JD (1993): Gender preselection in humans? Flow cytometric separaton of X and Y spermatozoa for the prevention of X-linked diseases. Hum Reprod 10:1733–1739.

Johnson LA, Welch, GR, Garner DL (1994): Improved flow sorting resolution of X- and Y- chromosome bearing viable sperm separation using dual staining and dead cell gating (abstr). Cytometry 7:83 (Abstract).

Katz DF, Overstreet JW (1981): Sperm motility assessment by videomicrography. Fertil Steril 35:188–193.

Owen DH, Katz DF (1993): Sampling factors influencing accuracy of sperm kinematic analysis. J Androl 14:210–221.

Sarkar S, Jolly, DJ Friedmann T, Jones OW (1984): Swimming behavior of X and Y human sperm. Differentiation 27:120–125.

SAS Institute Inc. (1994): "SAS/STAT User's Guide," Version 6, Vol. 2. Cary, NC, pp 891–996.

Suarez SS, Katz DF, Owen DH, Andrew JB, Powell RL (1991): Evidence for the function of hyperactivated motility in sperm. Biol Reprod 44:375–381.

Suttiyotin P, Thwaites CJ (1992): Comparison of a swim-up techniques with the Hamilton-Thorn motility analyser for measurement of sperm velocity and motility. Reprod Fertil Dev 4:153–160.

Welch GR, Jackson J, Waldbeiser G, Wall RJ, Johnson LA (1993): Single cell sorting and PCR to confirm separation of X and Y chromosome bearing sperm. Anim Biotech 6:131–139.

Yi Lui D, Clarke GN, Baker GHW (1991): Relationship between sperm motility assessed with the Hamilton-Thorn motility analyzer and fertilization rates in vitro. J Androl 12:231–239.