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# SEX RATIO AFTER INSEMINATION OF BOVINE SPERMATOZOA ISOLATED USING A BOVINE SERUM ALBUMIN GRADIENT<sup>1</sup>

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## Summary

This experiment was undertaken to determine if a method reported to successfully enrich the proportion of Y-chromosome-bearing spermatozoa in human semen could be adapted for separation of bovine spermatozoa. Semen was collected from four Angus bulls and aliquots were either separated on discontinuous gradients of bovine serum albumin (BSA) or untreated before processing for cryopreservation. Two hundred seventy-one cows or heifers were assigned randomly to be artificially inseminated ( $20 \times 10^6$  sperm/insemination) with separated or unseparated spermatozoa. The proportions of male offspring were 45 and 54% after inseminations with separated or unseparated spermatozoa, respectively. In a second phase of the experiment, pooled semen from three Holstein bulls was either extended and frozen without separation or frozen after separation using the discontinuous BSA gradient. Separated and unseparated spermatozoa were analyzed by flow cytometry to determine the ratio of X- and Y-chromosome-bearing spermatozoa based on differences in DNA content. The ratios of X- and Y-bearing spermatozoa in separated or unseparated samples were indistinguishable. We concluded that the

separation method did not enrich the proportion of Y-bearing bovine spermatozoa.

(Key Words: Bovine, Spermatozoa, Sex Ratio, Sperm Separation, Albumin Gradient.)

## Introduction

Ericsson et al. (1973) reported that human Y-chromosome-bearing spermatozoa, identified by quinacrine staining, could be isolated by applying spermatozoa to a series of gradients containing increasing concentrations of serum albumin. Since that time, several contradictory reports of attempts to repeat the separation have appeared (Evans et al., 1975; Ross et al., 1975; Ferguson et al., 1976; David et al., 1977; Dmowski et al., 1979; Quinlivan et al., 1982). Beernink and Ericsson (1982) reported a significant increase in the proportion of male offspring (75% male) born to 91 women inseminated with spermatozoa isolated using serum albumin gradients.

Recently, Pinkel et al. (1982) described a method for measuring nuclear DNA content by flow cytometry that allows determination of the proportion of X- and Y-chromosome-bearing spermatozoa. The process that has been adapted for identification of X- and Y-bearing bovine spermatozoa (Garner et al., 1983) provides a faster, less-expensive alternative for determining the proportion of X- and Y-bearing bovine spermatozoa than determining the sex ratio of offspring.

The objective of this project was to determine if a discontinuous bovine serum albumin (BSA) gradient, similar to that used by Beernink and Ericsson (1982), could be used to isolate a population of Y-chromosome-bearing bovine spermatozoa as determined by flow cytometric measurement of DNA content and to determine if artificial insemination of cows with the separated spermatozoa could increase the proportion of male offspring.

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### Experimental Procedure

*Sperm Separation for Artificial Insemination.* Semen was collected with an artificial vagina from each of four Angus bulls. Bulls were AI sires and were on a routine collection schedule. The first and second ejaculates collected from each bull were pooled, but semen from individual bulls was handled separately. The number of spermatozoa in the pooled semen was determined by duplicate hemacytometer counts. Aliquots of semen were prepared for cryopreservation without separation (unseparated), or the semen was prepared for separation on a discontinuous gradient of BSA.

Unseparated semen was extended in egg yolk-citrate extender with slow, step-wise addition of glycerol (20% v/v egg yolk, 73% v/v .1 M sodium citrate buffer, pH 6.9 and 7% v/v glycerol). Unseparated, glycerolated semen was packaged in .5-ml French straws ( $20 \times 10^6$  spermatozoa/straw) and frozen in liquid nitrogen vapor.

Semen to be separated using the BSA gradient was extended in Tris buffer (.25 M Tris-hydroxymethyl-aminomethane, .07 M fructose, .08 M citric acid monohydrate; pH 6.8; 310 mOsmol) and 26 ml of extended semen, containing  $1 \times 10^9$  spermatozoa, was applied to each BSA gradient. Semen from each bull was applied to two, three or four gradients. The BSA gradients consisted of 60 ml of 4% BSA (2.4 g BSA<sup>5</sup>/60 ml Tris buffer; pH adjusted to 6.8) layered over 60 ml of 10% BSA (6.0 g BSA/60 ml Tris buffer; pH adjusted to 6.8) in a globe-shaped, 500-ml separatory funnel<sup>6</sup>.

After allowing 1 h for spermatozoa to migrate at room temperature (21 to 24 C), two 20-ml aliquots were collected from the bottom of the 10% BSA. Thirty milliliters of Tris-egg yolk buffer (20% v/v egg yolk) that had been clarified by passing through a .45- $\mu$ m pore cellulose filter were added to each aliquot and the diluted aliquots were cooled to 5 C over 1.5 h. After cooling, spermatozoa in the two aliquots were centrifuged

( $630 \times g$ , 10 min, 5 C) to form a soft pellet. The supernatants were removed and recentrifuged. The sperm pellets from the centrifugations were resuspended and combined with a small volume of Tris-egg yolk buffer (clarified). Spermatozoa that had been separated on two, three or four gradients were combined, within bulls. Duplicate hemacytometer counts were performed and each sample was extended in Tris-egg yolk buffer with slow, step-wise addition of glycerol (to 7% v/v). The separated semen was frozen in .5-ml French straws ( $20 \times 10^6$  spermatozoa/straw) in liquid nitrogen vapor.

After at least 3 wk of storage at  $-196$  C, three straws of semen from each bull and each semen treatment were thawed in a water bath at 37 C for 45 s, pooled and evaluated to determine the percentage of motile sperm cells. Subjective estimations of motility were made by placing a glass coverslip over thawed semen ( $\sim 5$   $\mu$ l) diluted in a drop ( $\sim 10$   $\mu$ l) of Tris buffer. Slides were warmed to 37 C and a phase contrast microscope (100 $\times$ ) equipped with a heated stage was used to estimate the percentage of progressively motile cells in two smears from each sample. Smears were coded to prevent biased estimations.

Two hundred seventy-one crossbred cows or heifers in two herds were assigned randomly in a ratio of 2:1 to be bred with either separated or unseparated semen, respectively. Semen from each sire and semen treatment was distributed randomly across herds, and qualified technicians were assigned to breed cows at random and without knowledge of the bull or semen treatment in question.

Sex ratios achieved after insemination with either separated or unseparated semen and the interactions between the effects of the semen treatments and the four sires were determined using chi-square analyses described by Steel and Torrie (1960).

*Sperm Separation for Flow Cytometry.* An attempt was made to analyze the DNA content of spermatozoa in the separated semen fractions used for artificial insemination. However, the Tris buffer used to extend the semen interfered with the washing-fixation-staining procedure used for flow cytometry and it became necessary to prepare another group of separated semen fractions for flow cytometry in which the separated spermatozoa were diluted in egg yolk-citrate-glycerol extender.

<sup>5</sup>Fraction V powder, Sigma Chemical Co., St. Louis, MO.

<sup>6</sup>Corning, no. 6340, American Scientific Products, McGaw Park, IL.

TABLE 1. PREGNANCY RATE AND SEX RATIO OF OFFSPRING OF COWS INSEMINATED WITH UNSEPARATED SPERMATOZOA OR SPERMATOZOA ISOLATED WITH A BSA GRADIENT

Bull	Unseparated spermatozoa				Separated spermatozoa			
	No. insemin.	Percentage pregnant	M:F <sup>a</sup>	Percentage males	No. insemin.	Percentage pregnant	M:F <sup>a</sup>	Percentage males
34	16	44	3:4	43	33	73	16:8	67
47	39	62	15:9	62	82	66	20:34	37
63	9	78	4:3	57	18	39	5:2	71
64	26	62	7:9	44	49	67	13:21 <sup>b</sup>	38
Overall	90	61	29:25	54	182	65	54:65	45

<sup>a</sup>M:F represents the ratio of male to female calves.

<sup>b</sup>One cow in this group gave birth to twin female calves.

Ejaculates from three Holstein bulls were pooled after collection with an artificial vagina. A portion of the pooled ejaculates was extended in egg yolk-citrate-glycerol, packaged in .5-ml French straws, and frozen without separation as described above.

Other portions of the pooled semen were extended in Tris buffer and either applied to the discontinuous BSA gradient as described, or simply diluted in 10% BSA (BSA control). After 1 h, semen collected from the 4% layer of BSA in the gradient, semen collected from the 10% layer of the BSA gradient, or semen diluted in 10% BSA for 1 h was extended and washed in egg yolk-citrate buffer. The semen was glycerolated, packaged and frozen as described above. Immediately after freezing, the semen was stored in liquid nitrogen and shipped to the Lawrence Livermore National Laboratory, Livermore, California for flow cytometric analysis. These samples were evaluated as part of a flow cytometric study to measure the effectiveness of several sperm separation procedures (Pinkel et al., 1983).

The DNA content of unseparated spermatozoa, spermatozoa recovered from the 4% or 10% layer of the discontinuous BSA gradient, or unseparated spermatozoa incubated in 10% BSA was measured by flow cytometry according to procedures described by Garner et al. (1983). The semen was thawed, washed in sodium citrate-dimethyl sulfoxide, fixed in 80% ethanol, treated with papain and dithioerythritol, and stained for DNA with

the flurochrome 4'-6 diamidino-2-phenyl-indole. The resulting sperm nuclei suspension was then measured for DNA content using an epi-illumination flow cytometer<sup>7</sup>.

#### Results and Discussion

The mean post-thaw motility of unseparated semen extended in citrate buffer and semen collected from the lower one-third of the discontinuous BSA gradient and extended in Tris buffer was  $35 \pm 11.5$  and  $60 \pm 8.2\%$ , respectively. Sixty-one percent of the cows inseminated with unseparated spermatozoa and 65% of the cows inseminated with separated spermatozoa calved.

The proportions of male calves born to cows inseminated with unseparated (54%) or separated semen (45%) were not different (table 1;  $P < .25$ ). The proportion of male calves also did not vary among sires (sire  $\times$  semen treatment,  $P < .10$ ).

In a similar experiment, Ericsson et al. (1980) attempted to isolate the Y-chromosome-bearing spermatozoa of Charolais bulls using a less-selective, one-step, serum albumin separation procedure. Insemination of bovine spermatozoa isolated using the one-step BSA gradient also did not consistently increase the proportion of male offspring.

The proportion of Y-chromosome-bearing spermatozoa was not increased in any of the semen samples analyzed for DNA content by flow cytometry (table 2). The estimated ratios of X- and Y-bearing spermatozoa in samples recovered from the upper (4% BSA) and lower (10% BSA) layers of the discontinuous BSA gradient were indistinguishable from the ratio measured in unseparated semen.

<sup>7</sup>ICP 22, Ortho Instruments, Westwood, MA.

TABLE 2. RATIO OF X- TO Y-CHROMOSOME BEARING SPERMATOZOA IN SAMPLES OF UNSEPARATED OR SEPARATED SPERMATOZOA BASED ON RELATIVE DNA CONTENT AS DETERMINED BY FLOW CYTOMETRY

Unseparated spermatozoa		Separated spermatozoa	
No treatment	BSA-exposure <sup>a</sup>	4% BSA layer	10% BSA layer
50:50	47:53	51:49	48:52

<sup>a</sup>Unseparated spermatozoa exposed to 10% BSA for 1 h.

Exposure to 10% BSA did not influence the measurement of DNA content.

Although the semen used for flow cytometry estimation of DNA content was not the same semen used for artificial inseminations, the results of both aspects of this investigation indicate that the discontinuous BSA gradient used in these experiments did not isolate a population of Y-chromosome-bearing spermatozoa.

In neither this investigation nor the experiments with cattle reported by Ericsson et al. (1980) was the sex ratio of the offspring influenced by sperm separated with a BSA gradient, as it was in women inseminated with separated human semen (Beernink and Ericsson, 1982). The BSA gradient used in the present investigation was a two-phase gradient (4%:10% BSA), whereas human spermatozoa were separated on a single-phase gradient (7.5% human serum albumin, HSA), washed, resuspended and reapplied to another two-phase gradient (12.5%:20% HSA). Despite differences in the gradient systems, both human spermatozoa and bovine spermatozoa collected from the layer of most concentrated BSA have a motility of greater than 90% before freezing and the number of spermatozoa recovered is less than 10% of the spermatozoa in the original semen samples (Beernink and Ericsson, 1982; White, 1982). Therefore, the selectivity of the methods used for separation of bovine or human semen appear to be similar.

Differences in motility of X- and Y-chromosome-bearing human spermatozoa have been reported (Goodall and Roberts, 1976), and the albumin gradient reportedly separates human X- and Y-bearing spermatozoa based on differences in motility (Ericsson et al., 1973). We were unable to enrich the proportion of Y-bearing spermatozoa using a discontinuous BSA gradient in this experiment. Although this casts doubt on the ability of

the BSA gradient to separate X- and Y-bearing spermatozoa, differences among species in the patterns of motility or differences in sperm surface characteristics that influence the interaction between the sperm cells and the serum albumin may be responsible for the differences in results.

#### Literature Cited

- Beernink, F. J. and R. J. Ericsson. 1982. Male sex preselection through sperm isolation. *Fertil. Steril.* 38:493.
- David, G., C. Jeulin, A. Boyce and D. Schwartz. 1977. Motility and percentage of Y- and YY-bearing spermatozoa in human semen samples after passage through bovine serum albumin. *J. Reprod. Fertil.* 50:377.
- Dmowski, W. P., L. Gaynor, R. Rao, M. Lawrence and A. Scommegna. 1979. Use of albumin gradients for X and Y sperm separation and clinical experience with male sex preselection. *Fertil. Steril.* 31:52.
- Ericsson, R. J., B. Cassou and G. Dapremant. 1980. Isolation of progressively motile mammalian sperm: To select for Y sperm or to improve fertility. *Proc. IX Int. Cong. Anim. Reprod. Artificial Insemination III*:286.
- Ericsson, R. J., C. N. Langevin and M. Nishino. 1973. Isolation of fractions rich in human Y sperm. *Nature* 246:421.
- Evans, J. M., T. A. Douglas and J. P. Renton. 1975. An attempt to separate fractions rich in human Y sperm. *Nature* 253:352.
- Ferguson, J. M., T. A. Douglas and J. P. Renton. 1976. Studies on the separation of X- and Y-bearing spermatozoa. *Brit. J. Obstet. Gynaecol.* 83:411 (Abstr.).
- Garner, D. L., B. L. Gledhill, D. Pinkel, S. Lake, D. Stephenson, M. A. Van Dilla and L. A. Johnson. 1983. Quantification of the X- and Y-chromosome-bearing spermatozoa of domestic animals by flow cytometry. *Biol. Reprod.* 28:312.
- Goodall, H. and A. M. Roberts. 1976. Differences in motility of human X- and Y-bearing spermatozoa. *J. Reprod. Fertil.* 48:433.
- Pinkel, D., D. L. Garner, B. L. Gledhill, S. Lake, D. Stephenson and L. A. Johnson. 1983. The proportions of X- and Y-chromosome-bearing spermatozoa in samples of purportedly enriched bovine semen. *J. Anim. Sci.* 57 (Suppl.

- 1):366.
- Pinkel, D., S. Lake, B. L. Gledhill, M. A. Van Dilla, D. Stephenson and G. Watchmaker. 1982. Flow cytometric DNA content measurements of mammalian sperm. *Cytometry* 3:1.
- Quinlivan, W.L.G., K. Preciado, T. L. Long and H. Sullivan. 1982. Separation of human X and Y spermatozoa by albumin gradients and sephadex chromatography. *Fertil. Steril.* 37:104.
- Ross, A., J. A. Robinson and H. J. Evans. 1975. Failure to confirm separation of X- and Y-bearing human sperm using BSA gradients. *Nature* 253:354.
- Steel, R.G.D. and J. H. Torrie. 1960. *Principles and Procedures of Statistics*. McGraw-Hill Book Co., New York.
- White, L. M. 1982. Attempts to isolate highly viable, morphologically normal, Y-chromosome-bearing bovine spermatozoa. M.S. Thesis. Virginia Polytechnic Institute and State Univ., Blacksburg.