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FRACTIONATION OF BOVINE SPERMATOZOA FOR SEX SELECTION: A RAPID IMMUNOMAGNETIC TECHNIQUE TO REMOVE SPERMATOZOA THAT CONTAIN THE H-Y ANTIGEN

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ABSTRACT

A study was conducted to rapidly fractionate bovine spermatozoa on the basis of cell-surface H-Y antigen (i.e., Y chromosome-bearing spermatozoa). A novel, rapid immunomagnetic method was developed for removal of spermatozoa that bound to anti-H-Y IgG. Fluorescent labeling and flow cytometry were used to measure the efficiency with which spermatozoa binding to anti-H-Y were removed by the immunomagnetic technique. Washed bovine spermatozoa (n=7 bulls) were treated with a mouse monoclonal IgG antibody to H-Y antigen (MoAb12/49). Fluorescent labeled goat antibody against mouse IgG was added to label those spermatozoa with cell-surface H-Y antigens. Supermagnetized polymer beads coated with an anti-antibody to the MoAb 12/49 were then added to the spermatozoa. After 20 min of incubation, spermatozoa were exposed for 2 min to a magnet, causing the magnetized particles to adhere to the sides of the tube. Nonmagnetized spermatozoa in the supernatent were aspirated and analyzed for fluorescent label by flow cytometry. Approximately 50% of spermatozoa not subjected to immunomagnetic separation were fluorescent labeled, and about one-half of the spermatozoa were observed microscopically to be bound to the magnetized polymer beads prior to magnetic separation ($P<0.05$). Following magnetic separation, only 1.2% ($P<0.05$) of the spermatozoa in the magnetic supernatent were fluorescent labeled. Assuming that only Y chromosome-bearing spermatozoa have cell-surface H-Y antigens, the present immunomagnetic fractionation removed almost all of the Y chromosome-bearing spermatozoa, leaving a population that was greater than 98% X chromosome-bearing spermatozoa.

Key words: bovine spermatozoa, chromosome, H-Y antigen, immunomagnetic cell sorting, flow cytometry

INTRODUCTION

Technological advances in the field of dairy cattle reproduction have increased genetic improvement resulting in an increased number of offspring for progeny testing programs. The rate of genetic improvement, can be further enhanced if more female progeny are available for

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testing. A sex selection procedure before conception occurred would produce female offspring at a faster rate. Despite extensive research, such a practical sex selection method remains elusive. Several techniques to sex spermatozoa have been described; however, they have for the most part been discounted. Recently, new sex preselection techniques, including discontinuous density gradient centrifugation (9) and detection of fluorescent bodies representing the Y chromosomes (13) were utilized in determining the sex of mammalian spermatozoa before conception had occurred.

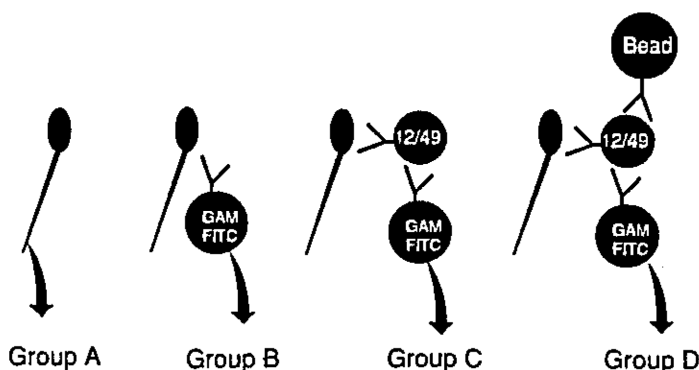
One approach which has been used successfully by several researchers in recent years is the separation of X and Y chromosome-bearing spermatozoa by flow cytometry (1,5,7,8,11,12). Although this technique provides excellent separation of X and Y chromosome-bearing populations, its high cost and slow rate of separation (~10^7 cells/h), as well as reports that the technique itself may be damaging to mammalian chromosomes, makes flow cytometry unlikely to become a commercially practical method (10).

The purpose of this study was to develop a rapid, inexpensive and commercially practical method for separating X and Y chromosome-bearing spermatozoa. Super-magnetized polymer beads coated with antibodies have been used to isolate several cell types, including cancer cells, leukocytes and human spermatozoa (4). Using these magnetic beads in conjunction with monoclonal antibodies against the male-specific H-Y antigen, an attempt was made to separate the bovine spermatozoa into X and Y chromosome-bearing fractions. The efficacy of the immunomagnetic method of separation was subsequently determined by flow cytometry.

MATERIALS AND METHODS

Handling of Semen

Frozen semen straws from 7 Angus bulls (aged 3 to 5 yr, Steward Angus, Indianapolis) were thawed in a water bath at 35°C for 60 sec and centrifuged at 900 x g for 4 min. The supernatant was discarded and the spermatozoa pellet was resuspended in 35°C phosphate-buffered saline (PBS) with a pH of 7.1 and centrifuged at 900 x g for 4 min. The washing procedure was repeated 3 times. After the final wash, the pellet was resuspended in PBS, and the sperm concentration for each sample was determined with a hemacytometer. Each bull's semen sample was divided into aliquots (n=4) that were assigned to 1 of the 4 experimental groups shown below.



Group A served as an unlabeled control. The spermatozoa were treated similar to that of Group C (see below) except that antibodies (primary and secondary antibodies) were not added.

Group B served as a control for nonspecific binding of the secondary antibody. The spermatozoa were treated similar to that of group C except that the primary antibody was not added.

Group C consisted of spermatozoa bearing the H-Y antigen and were labeled, as described (1), with slight modifications. The primary antibody was a monoclonal mouse immunoglobulin (designated 12/49) against H-Y antigen. This anti-H-Y IgG was generously supplied by G. C. Koo (Merck and Company) and was described previously (1). Briefly, 10 µl of a 1:32 dilution of anti-H-Y (12/49) were added to 10^6 cells suspended in 250 µl PBS and allowed to incubate for 20 min at room temperature. The samples were gently vortexed at the time the primary antibody was added and again after 10 min incubation. Following incubation, 1 ml of PBS was added to each sample, and the samples were centrifuged at 900 x g for 4 min. The supernatant was discarded, and the spermatozoa pellet was resuspended in one ml of PBS and washed again at 900 x g for 4 min. Each pellet was resuspended in 250 µl of PBS and 10 µl of a 1:8 dilution of the secondary antibody was added. The secondary antibody was a goat antibody to mouse IgG that was labeled with fluorescein-isothiocyanate. The samples were gently vortexed and allowed to incubate in the dark for 20 min at room temperature.

Group D spermatozoa were identical to those of Group C except that washed super-magnetized polymer beads coated with an anti-antibody to the MoAb 12/49 (Dynabeads-M450, Dynal INC) were added to each sample at a ratio of 40 beads per cell (40×10^6 beads) at the same time the secondary antibody was added. The samples were examined with a light microscope. One hundred spermatozoa were counted for each sample to determine the percentage of cells associated with beads. The samples were then exposed to a magnet (MPC-1, Dynal INC) for 2 min. The magnet caused the magnetized particles to adhere to the sides of the test tube, allowing the supernatant to be easily aspirated by pipette.

Flow Cytometry

Immunomagnetically sorted spermatozoa in each group were evaluated by flow cytometry (Epics Elite, Coulter Electronics, Hialeah, FL) for the presence of fluorescence. A population of 10,000 spermatozoa was used from each group for flow cytometry evaluation. The flow cytometer counted the number of cells registering fluorescence and reported the number as a percentage for each population.

Data Analysis

The probabilities of significant differences in the percentage of spermatozoa showing fluorescence between the groups were tested by one-way analysis of variance using the general linear model option of SAS (2). Differences between treatment means were tested by Duncan's multiple comparison test (14). Values of $P > 0.05$ were not considered to be significant.

RESULTS

Spermatozoa from Groups A, B and C were not attached to the beads (Figure 1). Microscopic evaluation of Group D spermatozoa showed that approximately 50% of the spermatozoa were in contact with one or more beads. Dynabead attachment generally occurred on the head or midpiece of the spermatozoa (Figure 2).

Flow cytometry data revealed that immunomagnetic sorting successfully removed more than 98% of the fluorescent-labeled spermatozoa from each sample (Table 1).

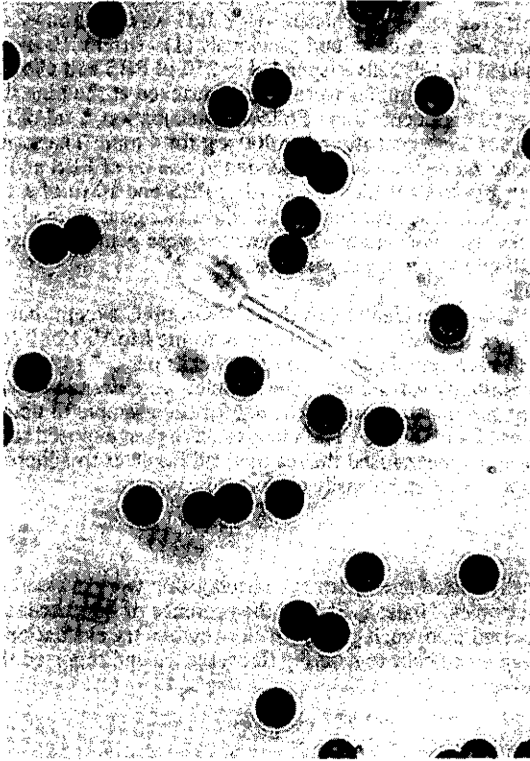


Figure 1. Spermatozoa not attached to super-magnetized polymer beads.

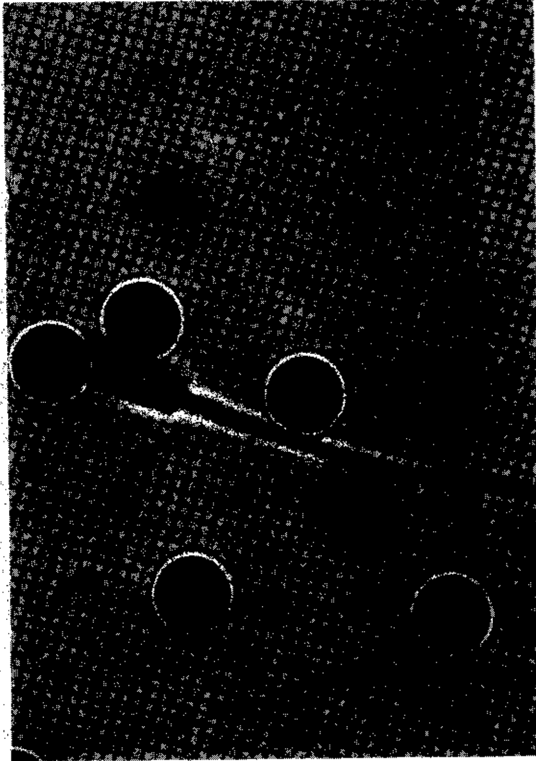


Figure 2. Spermatozoa attached to super-magnetized polymer beads.

Figure 3. Typical fluorescence observed in Group C spermatozoa after the treatment.

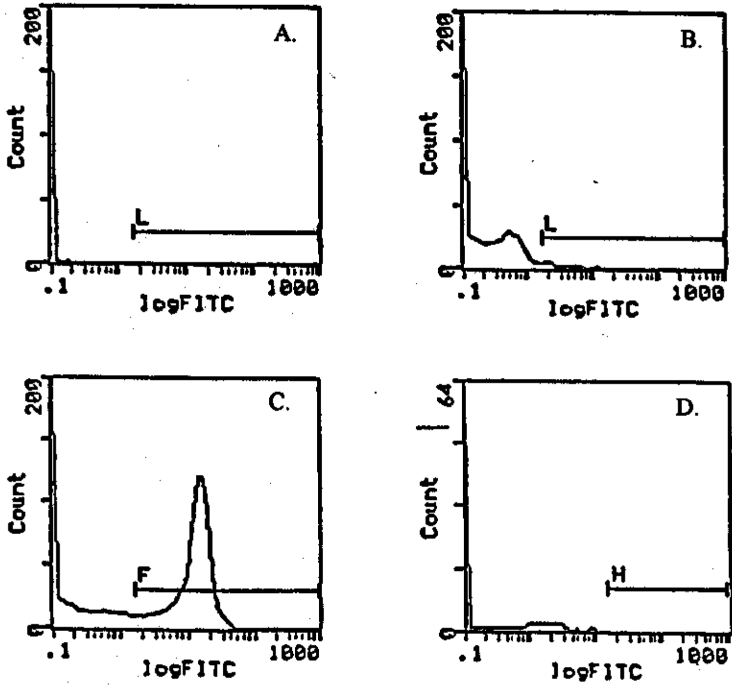


Figure 4. The flow cytometry histograms represent the 4 experimental groups (A-D). Each histogram represents a population of 10,000 spermatozoa.

Table 1. Percentage of spermatozoa-showing fluorescence by flow cytometry or attachment to dynabeads by light microscopy

ID	Group A[a]	Group B[b]	Group C[c]	Group D Pre-sorting[d]	Group D Post-sorting[e]
Bull 1	0.0	2.0	48.7	50	1.5
Bull 2	0.1	1.9	56.1	51	0.9
Bull 3	0.1	2.0	54.0	52	0.6
Bull 4	0.1	2.0	58.5	49	1.1
Bull 5	0.1	1.9	54.3	49	1.4
Bull 6	0.1	1.9	56.3	53	0.7
Bull 7	0.1	2.0	51.8	48	1.2

[a] Unlabeled control
[b] Fluorescein-isothiocyanate control
[c] % fluorescent spermatozoa prior to immunomagnetic sorting
[d] % of group D cells attached to beads prior to immunomagnetic sorting (determined by light microscopy)
[e] % fluorescent spermatozoa following immunomagnetic sorting

The average percentage fluorescent spermatozoa for each of the 7 bulls tested in the 4 groups was as follows: Group A (untreated spermatozoa) 0.1%; Group B (fluorescein-isothiocyanate control) 1.9%; Group C (treated spermatozoa pre-sorting) 51.8%; and Group D (Treated spermatozoa post-sorting) 1.2%. Figure 3 represents a typical fluorescence observed in Group C spermatozoa after the treatment. Representative flowcytometry histogram for each group is given in Figure 4.

DISCUSSION

Dynabead attachment generally occurred on the head or midpiece of the spermatozoa. These findings concur with other reports on the location of the H-Y antigen on spermatozoal membranes (6). Binding of anti H-Y antibodies to the post-acrosomal region of bull spermatozoa has also been reported (1,3)

The efficient separation of fluorescent-labeled spermatozoa by the immunomagnetic procedure was validated by flow cytometry. Approximately one-half of the spermatozoa in Group C (flow cytometry) and in pre-sort Group D (light microscopy) reacted with anti-H-Y, and there were less than 2% of these spermatozoa after immunomagnetic sorting (Table 2). This result combined with a previous finding of the H-Y antigen being specific to Y-bearing spermatozoa and the prior use of this particular anti-H-Y immunoglobulin (1) indicate that the present immunomagnetic technique produces an effective and practical separation of X chromosome-bearing and Y chromosome-bearing spermatozoa. If the fluorescence is specific for Y chromosome-bearing spermatozoa as hypothesized, this technique would produce nearly pure populations of X chromosome-bearing spermatozoa. Further studies are planned to determine the DNA content of the sorted spermatozoa (5,7) to provide further validation for this separation technique. Morphological changes to the spermatozoa due to the procedure were not studied in the present experiment. Similarly, the fertilizing capacity of the immunomagnetically sorted spermatozoa is not known, and experiments are underway to determine this ability. By these and other related studies to be conducted in the future, the applicability and practicality of this method under field conditions will be further elucidated. Definitely, much work needs to be conducted before a rapid, inexpensive and commercially practical method can be developed.

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