Production of Bovine Calves Following Separation of X- and Y- Chromosome Bearing Sperm and *In Vitro* Fertilization

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The capacity to produce cattle of predetermined sex would be of considerable benefit both from an economic and management viewpoint. Numerous attempts have been made seeking to separate X- and Y- chromosome bearing spermatozoa for use in sexing progeny (reviewed by Amann 1989, Gledhill 1988, Johnson 1992). However, to date no procedure has been devised and validated whereby sperm separation could be put into routine commercial artificial insemination. This is largely attributable to the lack of detectable differences in surface characteristics to achieve bulk separation and to the lack of suitably sensitive physical procedures able to exploit differences in mass.

To date the only procedure that has gained widespread acceptance as verifiably separating sperm from cattle, sheep and pigs is that which utilizes a flow cytometer/sorter as developed by Johnson and colleagues (Johnson and Pinkel 1986, Johnson 1991). The modified version of the flow cytometer is capable of distinguishing the small differences in DNA content between X- and Y-bearing sperm (some 3 to 4 per cent in the domestic species) after staining with the vital fluorochrome Hoechst 33342. Johnson has successfully applied flow sorting to the separation of viable sperm from the rabbit and pig (Johnson and others 1989, Johnson 1992) and following surgical insemination, normal young were born, the sex ratio of which was similar to the proportions (based on DNA) of the separated X- and Y-sperm used for the inseminations. No abnormalities of any kind were observed. In a study by Morrell and Dresser (1989) bovine, rabbit, porcine and ovine sperm were stained with Hoechst 33342 and passed through a flow cytometer and used for insemination. Out of a total of 292 births from the four species there was no evidence of gross anatomical abnormalities.

A modification of the procedure described by Johnson and Pinkel (1986) has been used to sort viable bull sperm for *in vitro* fertilization. A fluorescence activated cell sorter fitted with an ultraviolet laser (FACStar Plus; Becton Dickinson) was modified by the addition of

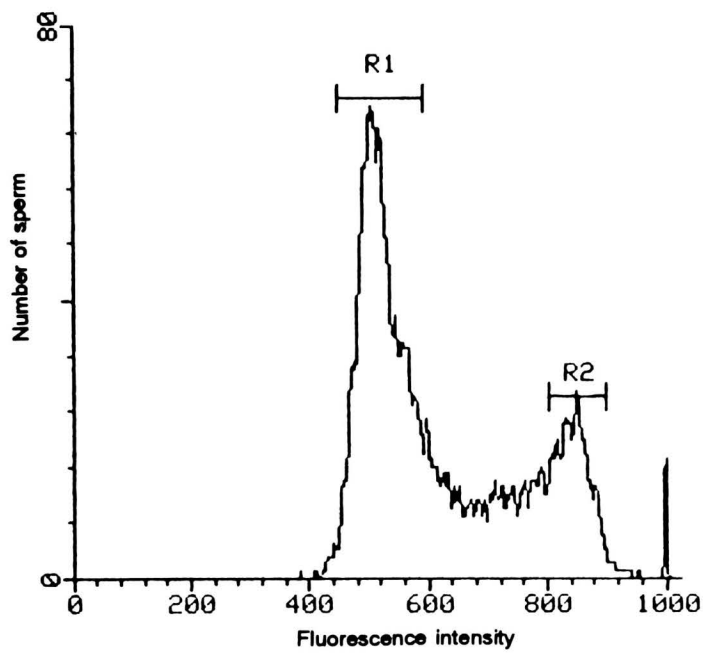

Figure 1: Histogram from the 90° detector showing the presence of a population of sperm (R2) whose edges are towards the 90° detector and whose faces are towards the 0° detector and the direction of propagation of the laser beam.

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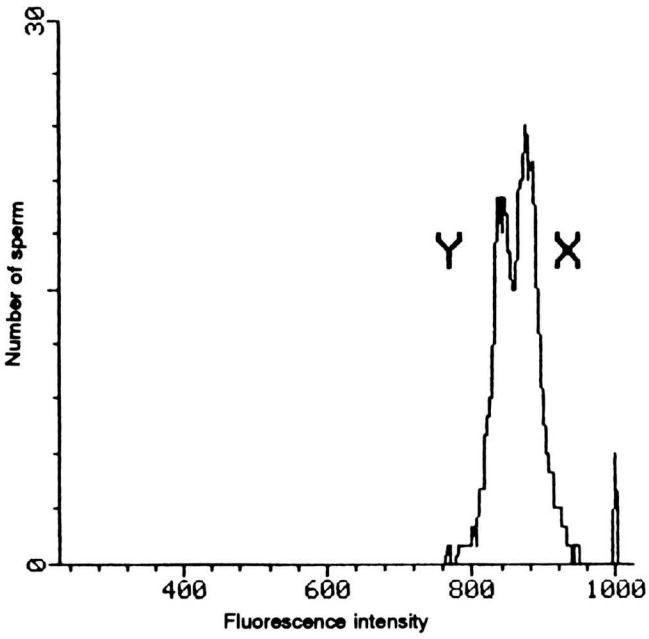

Figure 2: Histogram of signals obtained by the 0° detector of the gated region R2 in Fig. 1. The X-and Y-bearing populations are labelled.

two photomultiplier tubes (PMT) placed at right angles to each other. Due to their highly asymmetric shape a measure of the difference between X- and Y-bearing sperm can only be made for sperm passing with their flat faces parallel to a PMT and orthogonal to the laser beam. This PMT is described as the 0° detector. Due to their flat paddle shape and condensed DNA, irradiated sperm emit most light from their edges. This is detected by the second PMT (90° detector), and was used to detect those sperm in the appropriate orientation to the 0° detector for the resolution of the X- and Y-bearing populations. A histogram of the fluorescence as seen by the 90° detector showed two peaks with the oriented population to the right (Fig. 1). Fig. 2 shows the 0° image of the orientated sperm. Both X-and Y-bearing populations are clearly resolved. Sort gates were placed on these and the cells sorted into tubes containing Test buffer with varying percentages (10 to 20 per cent) of hen's egg yolk as described by Johnson and others (1989).

Sperm were stained with 9.0 μM Hoechst 33342 and sorted at a rate of some 100 per second to give a total of some one million X- and Y-bearing sperm used to carry out *in vitro* fertilization of *in vitro* matured eggs (Lu and others 1987). Reanalysis of the separated X- and Y-sperm for DNA in initial studies indicated that the purity of the X-sperm was 79 per cent and the Y-sperm 70 per cent. Embryos resulting from *in vitro* fertilization were sexed using a Y-probe (Miller and Koopman 1990) and amplification of the DNA product by the polymerase chain reaction (PCR) (Schroder and others 1990). PCR indicated that 73 per cent were female and 69 per cent male, a result not statistically different from that found for the sperm DNA analysis.

Twin embryo transfers were carried out on nine heifers four of which became pregnant resulting in the birth of three male and three female calves which had developed from eggs fertilized by Y-and X-sorted sperm, respectively. These initial results demonstrate that viable bovine sperm can be separated into X- and Y-bearing populations at a reasonably high purity using a cell sorter, that they retain their capacity for *in vitro* fertilization, that the sex of the resultant embryos is markedly skewed, and normal progeny can be produced. At present the number of X- and Y-bearing sperm that can be separated in the manner described is too low for artificial insemination and their use for *in vitro* fertilization is currently the only procedure available to produce embryos with a predetermined sex. It is to be expected that much higher purities will be obtained in the future with an accompanying increase in embryonic sex bias.

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