Flow Cytometric Sorting of Sperm: Influence on Fertilization and Embryo/Fetal Development in the Rabbit

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ABSTRACT

Viable, intact rabbit sperm, prepared, processed, and flow cytometrically sorted, were used in this study to determine the influence of flow sorting on fertilization and embryo development. In experiment I, flow-sorted or control (unstained and unsorted) sperm were surgically inseminated into the uterine horn of hormonally primed does (10 to 12 does per time point). At 42 hr postsurgical insemination, flushed embryos were assessed for development. Fetal development was determined at day 7, day 14, and day 21 post-surgical insemination. Embryos resulting from does surgically inseminated with control sperm at 42 hr post-insemination were observed to be at the early morula stage of development (>16 cell), whereas embryos from does inseminated with flow sorted sperm were at the 8- to 16-cell stage. No difference was observed between treatments at day 7, 14, or 21, however, there was a significant decrease in fetus number per doe inseminated with flow-sorted sperm over time. In experiment II, mature oocytes were flushed from the oviducts of superovulated does and coincubated in vitro (IVF) with flow-sorted or control rabbit sperm. Oocytes observed at 6 hr post-coincubation exhibited swollen sperm heads in the cytoplasm, demonstrating that fertilization had occurred (2PN + T). There was a higher percentage of fertilized oocytes by 8 hr post-coincubation for both control (31%) and flow-sorted sperm (31%) when used for IVF. By 10 and 12 hr post-coincubation, little difference was observed in the number of fertilized oocytes between sperm treatments (52% and 66% for control vs. 57% and 54% for flow-sorted, respectively). These studies demonstrate that flow-sorted sperm are capable of fertilizing mature oocytes under in vitro conditions. In addition they show that flow sorting may not negatively influence fertilization events, but likely interferes during early embryonic and fetal development. © 1996 Wiley-Liss, Inc.*

Key Words: Flow sorting, Fertilization, Embryo, Fetus, Sperm

INTRODUCTION

The inherent difference in DNA content between X- and Y-chromosome bearing sperm has served as the basis for the development of a successful method to flow cytometrically sort viable sperm into two populations (Johnson et al., 1987, 1989; Johnson, 1991). This cell sorting method (for review see Johnson, 1992, 1994) is used to obtain flow-sorted populations of viable intact sperm, which can be used for insemination and fertilization experiments (Johnson et al., 1989; Johnson, 1991; Cran et al., 1993). Progeny have been produced from flow cytometrically separated X- and Y-chromosome bearing rabbit sperm that were surgically inseminated (Johnson et al., 1989). Flow-sorted sperm were deposited at the tip of the rabbit uterus prior to ovulation. At 40 hr post-insemination, embryos recovered from flushing excised oviducts were normal and had developed to the 8- to 16-cell stage. At kindling, sex ratios of the young were skewed (94% female and 86% male from insemination made from respective X and Y sorted sperm) as predicted by laboratory validation of the sorted sperm for DNA. There was a reduction in kindling rate and litter size that was attributed to uterine manipulation during surgery, which was prior to ovulation or to the effects of the residual DNA-bound stain (Hoechst 33342). Altered sex ratios for live young have also been achieved in the pig (Johnson, 1991) using surgical insemination. Phenotypic sex ratios were skewed in the direction of male (68%) or female (74%) as predicted by analysis of DNA from aliquots of the flow-sorted sperm used for insemination. Litter sizes and farrowing rates obtained at the time of farrowing for gilts inseminated into the oviduct with sorted and stained sperm were lower than usual. Calves of the predicted sex have also been born using this flow-sorting procedure (Cran et al., 1993). In these studies, flow-sorted sperm were used for in vitro fertilization (IVF) and subsequent transfer of the embryo into recipient cows. From this initial study six calves were born, all of the predicted sex (Cran et al., 1993). In a followup field study designed to achieve a high percentage of males, 90% of the offspring were males (Johnson, 1994; Cran et al., 1995).

This study was designed to determine if the fertilization rate was affected by staining and sorting the sperm.

Received December 5, 1994; accepted June 19, 1995.
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and to determine at what point in time embryo/fetal development may be affected. The rabbit was selected as a representative model to test this question due to the ease of sorting sperm and ease of inseminating sperm into the uterus.

**MATERIALS AND METHODS**

**Sperm Preparation for Flow Cytometric Sorting and Surgical Insemination**

Semen was collected from mature New Zealand White bucks using an artificial vagina. Sperm concentration was determined with a hemacytometer and percentage of motile sperm estimated microscopically. Semen was diluted to $10 \times 10^6$ sperm/ml in a Tris buffer (0.21 M Tris, 58 mM glucose, and 67 mM citric acid; pH 6.9) and incubated with or without Hoechst 33342 (Calbiochem-Behring Inc., La Jolla, CA; HO42; 5 pg stain per $10^6$ sperm) for 1 hr in a 35°C heating block (Johnson et al., 1989). In an experiment conducted in a previous study, results obtained indicated that stained, unsorted sperm yielded fertilization rates similar to control (Johnson et al., 1991). Therefore, this treatment will not be included in this set of experiments.

**Flow Cytometric Sorting**

The viable sperm sorting procedure was essentially that as described earlier (Johnson et al., 1989). The sheath fluid used for viable sperm sorting was 10 mM phosphate-buffered saline (PBS; 1.45 M NaCl, 8.1 mM Na$_2$HPO$_4$, 1.9 mM NaH$_2$PO$_4$), pH 7.2, containing 0.1% bovine serum albumin (fraction V, BSA; A-7906, Sigma Chemical, St. Louis, MO). Stained intact sperm were sorted on a modified EPICS V/753 (Coulter Corporation, Hialeah, FL) flow cytometer/cell sorter (Johnson and Pinkel, 1986) using 200 mW of multiline ultraviolet excitation from a 90-5 Argon Laser (Coherent, Inc., Palo Alto, CA). Sorted sperm were collected into 1.5 ml polypropylene microfuge tubes, previously coated with 1% BSA in PBS to prevent sperm adherence. A 100 µl volume test extender containing 20% egg yolk was placed onto the bottom of each collection tube prior to sorting. No attempt was made to obtain highly purified X- and Y-chromosome bearing sperm populations in this study. After a 20 min settling period to allow concentration of the motile sperm in the lower portion of the tube, the supernatant was removed leaving a 250 µl volume of sample for insemination.

**Experiment I**

**Animal preparation and surgical insemination.** For experiment I, New Zealand White virgin does were induced to ovulate by intravenous injection of 300 IU human chorionic gonadotropin (hCG; LyphoMed Inc., Rosemont, IL). Approximately 6 hr post-hCG injection, surgical insemination was performed on anesthetized rabbits by exposing the uterine horn via laparotomy and 300,000 sperm in a 125 µl volume were placed at the tip of the uterus using a 1 cc syringe and 21-gauge needle (Johnson et al., 1989).

**Embryo and fetus evaluation.** Embryo and fetal development were assessed at 42 hr, and 7, 14, and 21 days post-surgical insemination (10–12 does were used per time point; each doe represents one replicate). Preliminary embryos were collected by flushing excised oviducts with a blunt 18-gauge needle and 12 cc syringe. Each oviduct was flushed with 6 ml PBS supplemented with 1 mg polyvinyl alcohol (P-8136 Sigma)/ml into a 60 × 15 mm Petri dish. Petri dishes were scanned using a dissecting scope and assessed for development. Harvested embryos were classified into four groups: unfertilized, embryos containing 1–4 cells, 8–16 cells, and early morula. Morphology of the blastomeres was documented. At 7 days post-insemination, uterine horns were examined for implantation sites. Fetus number, length, and development were assessed at days 14 and 21. The number of degenerating fetuses was also noted.

**Experiment II**

**Sperm preparation for IVF.** Semen was collected as described earlier. Fresh ejaculated semen was washed by centrifugation (350g, 5 min) in a 1:10 ratio with Tris buffer. Washed sperm were diluted to $10 \times 10^6$ sperm/ml with a high ionic strength-defined medium (370-375 mOsm, HIS-DM; Brackett and Oliphant, 1975) and incubated for 15 min at 38.5°C, 5% CO$_2$ to initiate capacitation. After incubation, 3 ml defined medium (DM; Brackett and Oliphant, 1975) was added to the incubation tube and sperm pelleted by centrifugation. The resulting sperm pellet was resuspended in 1 ml HIS-DM to yield a final concentration of $10 \times 10^6$ sperm/ml. This treatment served as the control. Fresh unwashed semen was prepared and processed for flow cytometric sorting as described earlier. Approximately $3 \times 10^6$ sperm were collected by flow sorting into a 4.7 ml polypropylene conical tube, previously coated with 1.0% BSA in PBS, containing 100 µl HIS-DM. Sorted samples were pelleted by centrifugation (350g, 5 min) and incubated in 300 µl HIS-DM for 10 min at 38.5°C, 5% CO$_2$ in an air incubator. After incubation a 1 ml volume of HIS-DM was added to the incubation tube and centrifuged for 5 min (350g) to pellet sperm. The sperm pellet was resuspended in 300 µl HIS-FM and held in the incubator until insemination. Samples of control and flow-sorted sperm were taken at the time of insemination and assessed for percent motile sperm and acrosome integrity using phase differential interference microscopy.

**Oocyte recovery and preparation for IVF.** In experiment II, New Zealand White virgin does were superovulated by administration of six subcutaneous injections of follicle-stimulating hormone (FSH; 0.3 mg each), given every 12 hr. Does were induced to ovulate by an intravenous injection of hCG (300 IU), administered 12 hr after the last FSH injection. In vivo matured oocytes were flushed from the oviduct 13 to 14 hr post-hCG injection via a cannula placed into the am-
pulla and inserting an 18-gauge blunt needle into the uterus and through the utero-tubal junction. Each side was flushed with 10 ml HIS-DM. Harvested oocytes from a group of four to six does were pooled and cumulus cells were removed from the oocytes by hyaluronidase treatment (0.1%; H-3506, Sigma). As dissociation of the cumulus cells from the oocyte began, oocytes were immediately removed and washed twice in HIS-DM. Washed oocytes were randomly assigned to 100 μl microdrops under silicone oil (Dow Corning 360 Medical fluid, 20 centistokes, Dow Corning Medical Products, Midland, MI) in a 35 × 10 mm Petri dish.

**IVF.** A 10 μl aliquot of sperm suspension (control or flow sorted) was added to each microdrop (1 × 10⁶ sperm/ml final concentration) containing 5–10 oocytes and the Petri dish placed into a 38.5°C, 5% CO₂ in air incubator (Kim et al., 1989). At 6 hr post-coincubation, oocytes were washed and cultured in TCM 199 (M-5017, Sigma) supplemented with 10% heat-inactivated fetal calf serum (Gibco Laboratories, Grand Island, NY) and 25 mM NaCO₃ and 1.25 mM pyruvate. At specified time points, oocytes were removed from culture and prepared for analysis. Samples were taken at 6, 8, 10, and 12 hr post-coincubation. To remove residual cumulus cells and sperm, oocytes were pipetted into a 15 ml conical tube containing 2 ml HIS-DM and vortexed for 1–2 min. After vortexing, the tube contents were poured into a 60 × 15 mm Petri dish and the tube rinsed with an additional 6 ml HIS-DM. Petri dishes were scanned, oocytes removed, placed onto a microscope slide, and fixed in acid alcohol. Fixed oocytes were allowed to clear for 24 hr, stained with aceto-orcein, and assessed for fertilization (Sirard et al., 1988).

**Statistical analysis.** Data were analyzed by the general linear model procedure using the Statistical Analysis System for Personal Computers (1992). Least square means were generated and used in the general linear model analysis. Stabilizing data variance for in vivo data was accomplished using arcsine transformation.

**RESULTS**

Analysis of preimplantation embryos from experiment 1 provided evidence that sorting influences development (Fig. 1). Embryos resulting from insemination with flow-sorted sperm and flushed at 42 hr post-surgical insemination had reached the 8- to 16-cell stage, whereas insemination with unstained, unsorted controls had reached the early morula stage of development. The percentage of early morula embryos was significantly greater when control sperm were used for insemination (P < .01). There was a slightly higher percentage of unfertilized oocytes when does were inseminated with flow-sorted sperm (24% vs. 15% for control sperm). Overall fertilization rates were higher for control sperm (84%) than for flow-sorted sperm (76%). Embryos obtained from surgically inseminated does, no matter the sperm treatment, were morphologically normal and microscopic observations revealed no apparent abnormalities.

Evaluation of reproductive tracts at day 7 of gestation revealed no difference (P < .05) in the number of implanted embryos between does inseminated with flow-sorted sperm or those inseminated with control sperm. Implantation number per rabbit inseminated with control sperm averaged 5.0 ± 0.3, whereas does inseminated with flow-sorted sperm averaged 3.0 ± 0.9 implantation sites per uterus (Fig. 2). However, by day 14 and 21 of gestation, there was an observed difference in the number of developing fetuses between treatments. Although the fetuses obtained from both groups
TABLE 1. Percentage of In Vivo Matured Oocytes Fertilized Using Control and Flow-Sorted Rabbit Sperm

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Sperm treatment</th>
<th>Total no. oocytes</th>
<th>Sperm penetration, oocyte not activated</th>
<th>Sperm penetration, oocyte activated</th>
<th>2 PN + T</th>
<th>PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Control</td>
<td>24</td>
<td>67%</td>
<td>10</td>
<td>23±0.3</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>Sorted</td>
<td>26</td>
<td>8%</td>
<td>8</td>
<td>25±0.3</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>Control</td>
<td>32</td>
<td>50%</td>
<td>7</td>
<td>12±0.3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Sorted</td>
<td>33</td>
<td>45%</td>
<td>0</td>
<td>3±0.3</td>
<td>56±0.3</td>
</tr>
<tr>
<td>12</td>
<td>Control</td>
<td>26</td>
<td>34±0.3</td>
<td>0</td>
<td>66±0.3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Sorted</td>
<td>26</td>
<td>38±0.3</td>
<td>4</td>
<td>54±0.3</td>
<td>4</td>
</tr>
</tbody>
</table>

*a* The abbreviation MII stands for metaphase II.
*b* The abbreviation 2PN + T represents two pronuclei plus tail.
*c* The abbreviation PS stands for polyspermy (described as oocytes fertilized with greater than one sperm).
*d* Values represent the number of total oocytes allotted to treatment and time for four replicates. (Each replicate represents one set of superovulated does.)
*+* Values within a column with different superscripts are significantly different (*P < 0.05*).

were developmentally normal, there were fewer fetuses obtained from both groups were developmentally normal, there were fewer fetuses per rabbit when flow-sorted sperm were used for surgical insemination (day 7, control sperm-6.0 ± 1.2 fetuses per rabbit vs. flow-sorted sperm, -2.0 ± 0.6 day 21, control sperm -7 ± 10 features per rabbit vs. Flow-sorted sperm -3.0 ± 0.9). Also, there was a higher number of degenerated fetuses when does were inseminated with flow-sorted sperm. Approximately 5% of the total number of fetuses evaluated were degenerative when flow-sorted sperm were used for insemination compared to less than 0.1% for control sperm. In general, there was a significant loss (*P < 0.05*) in the percentage of motile sperm (con- control sperm, 65.6 ± 1.8 vs. flow-sorted sperm, 58.1 ± 3.6) or percent- age acrosome intact sperm (control, 78.8 ± 1.5 vs. flow- sorted, 82.8 ± 1.3) between treatments. These results indicate that flow sorting rabbit sperm does not significantly influence subsequent fertilization events.

### DISCUSSION

The present study, utilizing in vivo and IVF methods, is the first attempt to address at what point of fertilization, and/or subsequent embryo development, affects may arise that can be attributed to the use of flow-sorted sperm. Previous work conducted in this laboratory has shown that kindling rates are lower and litter sizes smaller when flow-sorted sperm are used for insemination (Johnson et al., 1989; Johnson, 1991). However, it was unclear if these results were due to reproductive tract manipulation during surgery that interfered with oocyte pickup of if they could be due to the presence of residual vital stain (HO42) bound to DNA thus influencing fertilization and subsequent de-...
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velopment lagged in oocytes fertilized with flow-sorted sperm. In control animals, preimplantation embryos flushed from excised oviducts in experiment I were at the early morula stage of development (>16 cell) whereas oocytes cocultured with flow-sorted sperm were at the 8- to 16-cell stage (experiment II). The morphological characteristics of compaction, an event associated with the early morula stage embryo, had not yet occurred in the embryos fertilized with flow-sorted sperm. Compaction involves the cells moving into close proximity to maximize contact between blastomeres, thus forming a compact ball of cells. This arrangement is stabilized by tight junctions formed between outside cells, resulting in sealing off the interior of the sphere. Evidence suggests that compaction is mediated by events occurring at the cell surface of adjacent blastomeres and may involve specific cell surface proteins (Johnson et al., 1979; Hyafil et al., 1981; Peyrieras et al., 1983). The complement of the sperm genome to the zygote has not been defined, therefore one may postulate that any influence on sperm genomic material may influence events later in development. Studies investigating the effect of HO42 on cell function have shown that various cell types respond differently to HO42 staining (Wieszorak, 1984). The possible toxic properties of HO42 appear to depend on cell type and dye concentration used, with 1–10 μg HO42/ml being the typical concentration range in which no toxicity is observed in a variety of cell types (Conover and Gwatkin, 1988). However, a human tumor cell line (HT29), stained with a nontoxic concentration of HO42 commonly used in other cell types, displayed a ligand-induced cytotoxicity which caused a decrease in protein synthesis (Smith and Anderson, 1984). This supports the view that residual bound stain associated with flow-sorted sperm DNA may be a factor in early embryonic development by blocking DNA binding protein sites, thus interfering with protein synthesis. It is unclear what form of DNA damage may be occurring as sperm pass through the laser beam component of the flow cytometer. Sperm are in the path of the laser beam for less than 3 msec. Preliminary data suggest that reduced laser power increases embryo survival (unpublished). It is possible that UW radiation could induce slow embryonic development or loss of developing fetuses during gestation; however, we have not tested this hypothesis.

At day 7 of gestation, the time of implantation in the rabbit, there was little difference in the number of implantation sites per rabbit. Yet, by day 14, a noticeable loss in fetus number per rabbit was observed. Also, a higher percentage of degenerated fetuses were observed when flow-sorted sperm were used for surgical insemination. It is unknown what influence flow cytometric techniques may have on sperm that may later affect fetal development during gestation. Perhaps damage to the chromosomal material during flow sorting may interfere with information needed for regulation of events associated with development in some embryos.

Another possible consideration is that the lag in pre-implantation embryo development is due to an interference in early events of fertilization caused by flow-sorting sperm. The question then becomes: Does flow cytometric sorting of sperm affect sperm binding, nuclear decondensation, oocyte signalling, and/or the resumption of meiotic events in the maternal genome?

The ability of sperm to bind and penetrate the zona pellucida of mature metaphase II oocytes is the first important step to occur in the process of fertilization; followed by oocyte activation and sperm nuclear decondensation. In this study, an IVF experiment was conducted to address if these events of fertilization were being affected. Fertilization, under the culture conditions of this study, appears to occur around 6 hr coincubation. Pronuclear formation was first observed to occur at 8 hr post-coincubation. Also, there was a lower percentage of oocytes fertilized (12%) compared to control (20%) and activated when coincubated with flow-sorted sperm at 8 hr post-coincubation. These observations confirm the view that the process of flow cytometric sorting precipitates sperm (Johnson, 1991), therefore enabling fertilization to occur quickly after coincubation. However, precapacitation may also affect some sperm component associated with oocyte activation, thus influencing fertilization from the perspective or the oocyte. A sperm oocyte activation factor has been described for the rabbit, in which a protein or protein-like factor was able to induce oocytes to release cortical granules, form pronuclei, and cleave (Stice and Robl, 1990). It is unknown what influence flow cytometric sorting has on sperm membrane composition, Yet, by 10 and 12 hr coincubation, there was no significant difference in the number of fertilized oocytes at the pronuclear stage between control and flow-sorted sperm. These results suggest that flow-sorted sperm bind, penetrate, and decondense in a normal manner, as well as activate the oocyte to finish meiotic events. There was no significant indication that the lag in pre-implantation embryo development observed in the in vivo study is due to fertilization impairment, however, there is an occurrence of fertilized oocytes which is not activated when flow-sorted sperm are used for fertilization.

The data presented in this study provide information concerning the influence of flow cytometric sorting on sperm-fertilizing ability and embryo development. Flow-sorted sperm are capable of fertilizing mature oocytes under in vivo and in vitro conditions. However, some interference may occur during preimplantation development and later in fetal development. Since no morphological abnormalities have been observed in rabbits, swine, or cattle (Johnson et al., 1989; Johnson, 1991; Cran et al., 1993), when flow-sorted sperm are used, it is highly likely that all embryos that are slow to develop or that may be damaged die early in gestation and prior to reaching term. The data of Morrell and Dresser (1989) agree with these results in a study which gave no morphological abnormality in rabbits or cattle following insemination with flow-sorted sperm that had been passed in front of a laser beam after treatment with HO42.
ACKNOWLEDGMENTS
These studies were supported in part by Animal Biotechnology Cambridge, Ltd. (ABC), UK, under a Cooperative Research and Development Agreement 58-32U4-0-121 with the USDA-ARS. The authors gratefully acknowledge the important technical contributions of Glenn Welch and Cindi Garbus-Gooch and thank Judith Stephens, Lori Schreier, and Chris Gorum for assistance with surgeries and animal care.

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