Flow Cytometric Sperm Sorting: Effects of Varying Laser Power on Embryo Development in Swine


Germplasm and Gamete Physiology Laboratory, Agricultural Research Service, US Department of Agriculture, Beltsville, MD

ABSTRACT This study was conducted to determine fertilization rate and embryo development using the Beltsville Sperm Sexing Technology with two different laser power outputs, 25 and 125 milliwatts (mW). Freshly ejaculated boar semen was diluted; one aliquot was not stained or sorted (nonsort) and a second aliquot was stained with Hoechst 33342 and sorted as a complete population, not separated into X and Y populations (all-sort). Ovulation controlled gilts were surgically inseminated with 2 × 10⁵ spermatozoa (44–46 hr after human chorionic gonadotropin (hCG)) into the isthmus of each oviduct, one oviduct receiving nonsort and the other all-sort at 25 or 125 mW. A total of 426 embryos were flushed from oviducts at slaughter 43 hr after laparotomy and prepared for determination of fertilization and cleavage rates using confocal laser microscopy for analysis of actin cytoskeleton and chromatin configuration. The percentage of fertilized eggs and embryos was less for the 25 mW all-sort compared to nonsort or the 125 mW all-sort (77.9 vs. 96.3 and 96.2%, P < 0.05). The percentage of fragmented embryos was greater for the 25 mW all-sort than the nonsort (15.2 vs. 4.5%, P < 0.05), but did not differ significantly from 125 mW all-sort mean (7.2%). The percentage of normal embryos (80.4% overall) did not differ (P > 0.05) among treatments. However, the rate of embryo development was slower (P < 0.05) after insemination with the 25 mW all-sort spermatozoa compared to nonsort spermatozoa. Embryos in the 3–4 and 5–9 cell stages for the 25-mW all-sort and nonsort were 78 and 20% vs. 49 and 50%, respectively. The embryo percentages for the 125 mW (3–4 and 5–9 cell stages, 59 and 35%) did not differ significantly (P > 0.05) from the nonsort or 25 mW all-sort. We conclude that the use of 125 mW laser power for sorting boar spermatozoa is advantageous to maintain high resolution separation and has no detrimental effect on embryo development compared to 25 mW. Mol. Reprod. Dev. 61: 87–92, 2002.

Key Words: flow sorting; swine; sperm; fertilization; laser power

INTRODUCTION

Sex preselection has been a long sought goal of the livestock producer because it would provide animals of the desired sex and could result in a significant reduction in production costs. The only proven method of sex-preselection is the Beltsville Sperm Sexing Technology which is based on the relative difference in DNA content of the X- and Y-chromosome bearing spermatozoa. Sex-preselection using flow cytometric sperm cell sorting provides a significant shift in the 50:50 sex ratio of offspring to 90–95% male or female with precision and repeatability (Johnson et al., 1989, 1999; Johnson and Welch, 1999).

However, use of sex chromosome selected spermatozoa may affect embryo development. Insemination with sorted spermatozoa has resulted in reduced litter size in rabbits (Johnson et al., 1989) and swine (Johnson, 1991), and reduced embryo development rate in rabbits (McNutt and Johnson, 1996). Use of sorted bovine spermatozoa for in vitro fertilization reduced blastocyst development rate in culture (Cran et al., 1993). Sperm viability and acrosome morphology are reduced following the sort process compared to nonsorted spermatozoa (Maxwell and Johnson, 1997; Maxwell et al., 1997). The primary conclusion was that sorted sperm appear to be partially acrosome-reacted or pre-capacitated (Maxwell and Johnson, 1997; Maxwell et al., 1997). These changes may be related to the dilution of sperm in a medium that reduces the concentration of protective lipids and protein provided by seminal plasma (Mann, 1964; Maxwell et al., 1998) or involve factors associated with the sort process, such as, the exposure of Hoechst 33342 stained spermatozoa to 351 and 364 nm laser light, physical stress, and shear forces. The intensity of UV laser light is largely determined by the power output of the laser. In a previous study, the use of rabbit spermatozoa sorted at a laser power output of 200 mW resulted in a significant reduction of both fertilization and embryonic development compared to laser power output of 125 mW (Johnson et al., 1996). Beginning in 1996 we adapted our high-speed flow cytometer to the Beltsville Sperm Sexing Technology...
(Johnson and Welch, 1999; Johnson et al., 1999). High-speed sorting has not been completely characterized with respect to the effect of laser power output on fertilization and embryonic development. The present study was conducted to test the hypothesis that reduction of laser power output from 125 to 25 mW would attenuate reduction in fertilization and embryonic development following the sorting process applied to boar spermatozoa while maintaining other characteristics of laser operation constant (wavelength, exposure time, and beam dimension variables).

MATERIALS AND METHODS

Flow Sorting of Boar Spermatozoa

Semen was collected from mature boars on a regular twice weekly collection schedule using the gloved hand technique. Sperm were prepared for sorting using procedures described earlier (Johnson, 1991) as modified for high speed sorting (Johnson and Welch, 1999). Control (nonsort) aliquots were diluted in Beltsville TS (BTS; Johnson et al., 1988). Sperm for sorting were prepared as follows: an aliquot of 75 × 10^6 sperm/ml in BTS were treated with Hoechst 33342 (28 μM final concentration) and incubated for 1 hr at 35°C. Just prior to sorting, the stained sperm were treated with FD&C#40 food coloring (50 μM final concentration) for 5 min to quench the Hoechst-33342 fluorescence of dead sperm to differentiate them from the living sperm in the sample as described earlier (Johnson et al., 1999).

Sperm were subjected to the sorting process using a high speed MoFllo cell sorter (Cytomation, Inc., Ft Collins, CO) modified for sperm sorting (Johnson and Pinkel, 1986) including an orienting nozzle (Rens et al., 1998). Recent adaptations to high speed sorting of sperm have been described (Johnson and Welch, 1999). In this experiment the instrument sheath pressure was 40 psi and the event rate kept near 8,000 sperm/sec. The laser was a Coherent Inova 307 (Coherent, Inc., Palo Alto, CA) operating in the multi-line UV (351, 364 nm). Two different levels of laser power output 25 and 125 mW resulted in sort rates of 600–800 sperm/sec. The laser was verified using a remote, Coherent model 210, laser power meter. Spermatozoa sorted at 25 or 125 mW were used for the experiment. The laser power output was verified using a remote, Coherent model 210, laser power meter. Spermatozoa subjected to the sorting process were sorted as one population (all-sort) since sex predetermination was not a part of the experiment process were sorted as one population (all-sort) since sex predetermination was not a part of the experiment.

Synchronization of Estrus and Ovulation

The time of ovulation was controlled so that insemination was 2–4 hr after the expected time of ovulation. Eighteen crossbred gilts, weighing an average of 125 kg each, with at least two previous estrous cycles of 19–21 days, were synchronized by feeding 0.17 mg of the progesterone agonist, Altretrans (Roussel-Rousell Roussel Pharmaceuticals, Somerville, NJ) per kg body weight once daily for 7–10 days starting on day 11 to 13 of the estrous cycle. Follicle maturation was stimulated by subcutaneous injection of 1333 i.u. of equine chorionic gonadotropin (eCG; Diosynth, Chicago, IL) 22 hr after altrenogest withdrawal and ovulation was induced by i.m. of 1000 i.u. of human chorionic gonadotropin (hCG; Steris Laboratories, Phoenix, AR) 84 hr after eCG.

Surgical Insemination

At 44 to 46 hr after hCG, anesthesia was induced by administering the following per 100 kg body weight: 400 mg Ketamine HCl (Ketaset®, Aveco Co., Fort Dodge, IA); 200 mg xylazine (Rompun®, Haver Lockhart, Bayvet Division, Miles Laboratories, Shawnee, KS); 100 mg Telozol® (50 mg tileamine HCl and 50 mg xylazine tartrate (Turbugesic®, Fort Dodge Laboratories, Fort Dodge, IA); and 6 mg atropine sulfate (Butler Company, Columbus, OH). One-fifth of the anesthetic dose was administered i.m. and the remaining dose was administered i.v. after 10 min. During anesthesia each gilt’s reproductive tract was exteriorized at mid-ventral laparotomy and inseminated (Johnson, 1991) with 2 × 10^5 sperm into the isthmus of each oviduct. One oviduct received control nonsorted spermatozoa and the other oviduct received one of the two treatments, spermatozoa sorted at 25 or 125 mW. Animals used in this experiment were treated under an experimental protocol approved by the Beltsville Area Animal Care and Use Committee.

Embryo Analysis

Gilts were slaughtered by electrical shock and exsanguination 43 hr after laparotomy. Embryos were flushed from oviducts in Dulbecco’s PBS (Sigma Chemical Co., cat# D-1408, St. Louis, MO), fixed in 4% paraformaldehyde for 1 hr, and stored in PBS until analysis. Embryos were permeabilized with 1% Triton X 100, labeled with 2.5 U/ml (82.5 nM) Texas Red-X phalloidin (Molecular Probes, Eugene, OR), and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) containing 2 μg/ml Hoechst 33342. Confocal laser scanning microscopy was used to visualize the actin cytoskeleton and chromatin configuration in order to determine the incidence of fertilization, polyspermy, fragmentation, and cleavage. Embryos were observed using a Zeiss LSM 410 laser...
scanning confocal microscope (Carl Zeiss, Inc., Thornwood, NY). Texas Red-X was excited with the 568 nm line of a mixed gas Argon/Krypton laser and the emitted light was passed through a 590 nm long-pass filter; Hoechst 33342 labeled nuclear chromatin was excited with the 351 nm line of an Argon ion laser, and the emitted light was passed through a 397 nm long-pass filter. Images were obtained using a C- apo 40× water immersion objective (1.2 NA) at a zoom factor of 1.8. Each fluorochrome was scanned individually and the individual optical sections were pseudo-colored and digitally recombined into a single composite image using LSM software (Carl Zeiss, Inc.).

**Statistical Analysis**

Analysis of variance was performed using mixed model procedure in release 6.12 of the Statistical Analysis System software for personal computers (SAS, 1997). From each gilt, ovulation rate, total number of eggs and embryos, sperm penetration (sperm penetrated oocytes and embryos), normal embryos (3–9 cell embryos without polyspermy or fragmentation), normal 3–4 cell embryos, normal 5–9 cell embryos, polyspermic embryos, and fragmenting embryos (not polyspermic) were recorded and the percentage data were analyzed. Sperm treatment (nonsort, 25 mW, and 125 mW sort) was the fixed effect. Gilt was used as a random variable with compound symmetry covariance structure. Mean comparisons were made by the LSD procedure.

**RESULTS**

At slaughter, 426 potential embryos were flushed from oviducts; of these, 311 were embryos that were normal (cleaved without fragmentation or polyspermy), 3 were fertilized and uncleaved, 30 were fertilized and fragmented, and 43 were polyspermic. Of the 39 unfertilized oocytes recovered, the following stages were represented: germinal vesicle, 3; no DNA staining, 3; metaphase I, 3; and metaphase II, 30. Ovulation rate on the ovaries ipsilateral to each oviduct receiving spermatozoa did not differ significantly \( (P>0.05) \) among sperm treatments of non sort, 25 mW sort, and 125 mW sort, respectively (13.4, n = 18; 12.3, n = 9; and 17.1, n = 9). Egg and embryo recovery based on corpora lutea count also did not differ \( (P>0.05) \) among sperm treatments of nonsort, 25 mW sort, and 125 mW sort (86.8, 82.1, and 83.9%, respectively).

Figure 1 shows examples of eggs and embryos flushed from oviducts 44 hr after injection of hCG. The use of laser scanning confocal microscopy provided a very sensitive method of distinguishing between normal and abnormal embryos. Hoechst 33342 staining of nuclear chromatin enabled normal and polyspermic nuclei to be distinguished, while phalloidin labeling of the actin cytoskeleton allowed the discrimination of normal blastomeres and fragments (a structure resembling a blastomere that contained no nucleus). An unfertilized egg at the metaphase I stage of development is shown (Fig. 1A). A polyspermic 4-cell embryo is shown with two normal and two abnormal blastomeres that contained multiple polysegmented micro-nuclei (Fig. 1B) and a fragmented 4-cell embryo is shown which contains a blastomere without a nucleus (Fig. 1C). Two normal four-cell (Fig. 1D and E) and one normal eight-cell (Fig. 1F) embryos are also shown for comparison.

The effects of laser power output on embryo development are shown in Fig. 2. The percentage of embryos with sperm penetration (Fig. 2A) was lower for the 25 mW sort compared to nonsort and 125 mW sort (77.9 vs. 96.3 and 96.2%, respectively; \( P<0.05 \)). The percentage of normal embryos (Fig. 2B) did not differ among treatments (80.4% overall; \( P>0.05 \)). However, the percentage of 3–4 and 5–9 cell normal embryos varied among treatments. The percentage of 3–4 cell embryos (Fig. 2C) was greater for the 25 mW sort than for the nonsort (78.2 vs. 49.2%; \( P<0.05 \)); however the nonsort and 25 mW treatment means did not differ significantly from that of the 125 mW sort (59.0%; \( P>0.05 \)). In contrast, the percentage of 5–9 cell embryos (Fig. 2D) for the 25 mW sort was less than the nonsort (20 vs. 49.6%; \( P<0.05 \)); however the nonsort and 25 mW treatments did not differ significantly from that of the 125 mW sort (35.1%; \( P>0.05 \)). The incidence of polyspermic embryos (Fig. 2E) did not differ among spermatozoa treatments (11.1%; \( P>0.05 \)). The percentage of fragmented embryos (Fig. 2F) was greater for the 25 mW sort than the nonsort (15.2 vs. 4.5%; \( P<0.05 \)), however the nonsort and 25 mW treatments did not differ significantly from that of the 125 mW sort (7.2%; \( P>0.05 \)).

**DISCUSSION**

Improvements in the speed and efficiency of the Beltsville Sperm Sexing Technology since the birth of live offspring (Johnson et al., 1989; Johnson, 1991) have continued for several years (Rens et al., 1998; Johnson and Welch, 1999; Johnson et al., 1999). Earlier experiments to determine the effects of laser power changes on fertilization and embryo development were conducted with the standard speed sorter (Johnson et al., 1996). Based on those results in rabbits we hypothesized that sorting boar spermatozoa at a low laser power output (25 mW) would increase the rate of embryo development compared to sorting at a high laser power output (125 mW). The original protocols (Johnson et al., 1989) were developed using a laser power of 175–200 mW. In order to optimize the resolving power of the system for separating X from Y sperm with minimum laser power it was found that 75 mW of laser power gave significantly higher fertilization rates than 200 mW (Johnson et al., 1996). The use of our current high speed sperm sorter (Johnson and Welch, 1999) has made adequate resolution of X and Y sperm possible even at lower laser power (25 mW).

The results of the current study did not support our hypothesis that lowering laser power would have less impact on fertilization and embryo development. With
Fig. 1. Confocal images to show the embryo classification. A, unfertilized oocyte in MI stage of maturation; B, polyspermic 4-cell embryo (polyspermic nuclei marked by arrows); C, fragmenting 4-cell embryo (fragment marked by arrow), D, normal 4-cell embryo; E, normal 4-cell embryo; and F, normal 8-cell embryo.
In respect to fertilization, our data indicate that sperm cells sorted at 25 mW may be at a disadvantage after oviductal insemination compared to nonsorted and 125 mW all-sort spermatozoa. In previous studies, using standard or high speed sorter sperm cell sorting, no effect on fertilization rate was found in rabbits (McNutt and Johnson, 1996), cattle (Cran et al., 1993; Schenk et al., 1999), or in swine when in vitro fertilization conditions were optimized to accommodate sorted spermatozoa (Abeydeera et al., 1998; Rath et al., 1999). When spermatozoa from the 25 mW all-sort were used for fertilization, compared to nonsorted spermatozoa, the population of embryos shifted more toward 3–4 cell stages and away from 5–9 cell stages and the percentage of fragmenting embryos was increased. While the rate of embryo development after insemination with sperm sorted at 125 mW tended to be less than that of the nonsorted and greater than that of 25 mW spermatozoa, the differences were not statistically significant.

The first live offspring from sexed spermatozoa showed reduced litter size in rabbits and sows (Johnson et al., 1989; Johnson, 1991) indicating that fertilization and/or embryo survival were reduced as a result of
insemination with flow-sorted X or Y spermatozoa. Subsequently, McNutt and Johnson (1996) found that flow-sorting at 200 mW influenced the development of embryos flushed at 42 hr post-surgical insemination. Embryos produced after insemination with sorted spermatozoa that had reached the morula stage of development at a later time than embryos from unstained, unsorted spermatozoa. This effect on early embryo development resulted in lower embryo survival, which resulted in significantly fewer fetuses from flow-sorted sperm inseminations than for control-unsorted-unstained sperm inseminations. A similar effect was shown in cattle using sperm stained and sorted at 150 mW of laser power for in vitro fertilization. In that study there was a delay of 12 hr in blastocyst development when compared to the use of non-stained, nonsorted sperm (Cran et al., 1993).

The difference in embryo development in response to laser power output in this study and earlier studies mentioned above may be related to the fact that all the previous data were collected with standard speed sorter equipment similar to that described in the original report (Johnson et al., 1989). Although it is not known for sure, it is assumed that the UV laser light may be having an impact on some of the sperm that leads to reduced embryo survival. The duration of exposure of the stained sperm to UV laser light in the current high speed sperm sorter (MoFlo, Cytomation, Inc.) is approximately 1 μsec, 33–50% of the exposure time in the older standard speed Epics V 753 (Coulter Corporation, Miami, FL), consequently any presumed UV damage of spermatozoa would be expected to be less with shorter duration UV laser light exposure used for high speed sorting.

The reduction in fertilizing capacity, and calving and farrowing rates after insemination with sex sorted sperm is well established. While insemination with sperm sorted at low laser power output in this study did retard embryo development compared to nonsorted spermatozoa, it was not significantly different than embryo development with spermatozoa sorted at higher laser power. Regardless of the laser power setting used on standard or high speed sorters, one fact is established, i.e., rabbits, pigs, and cattle produced by insemination with sorted sperm are morphologically and reproductively normal (Johnson et al., 1989; Johnson, 1991; Cran et al., 1993, 1995; Rath et al., 1997, 1999; Seidel et al., 1997, 1999; Abeydeera et al., 1998). Rabbits and swine have also been reproduced through the third generation from sexed offspring with normal development (Johnson LA, unpublished data). We conclude that the use of 125 mW laser power for sorting boar spermatozoa is advantageous to maintain high resolution separation (Johnson and Welch, 1999) and has no detrimental effect on embryo development compared to 25 mW.

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REFERENCES


