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Developmental Potential of Vitrified Holstein Cattle Embryos Fertilized In Vitro with Sex-Sorted Sperm

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ABSTRACT

In vitro fertilization (IVF) is a feasible way to utilize sex-sorted sperm to produce offspring of a predetermined sex in the livestock industry. The objective of the present study was to examine the effects of various factors on bovine IVF and to systematically improve the efficiency of IVF production using sex-sorted sperm. Both bulls and sorting contributed to the variability among differential development rates of embryos fertilized by sexed sperm. Increased sorting pressures (275.8 to 344.75 kPa) did not have a significant effect on the in vitro fertility of the sorted sperm; neither did an extended period of 9 to 14 h from semen collection to sorting. As few as 600 sorted sperm were used to fertilize an oocyte, resulting in blastocyst development of 33.2%. Postwarming of vitrified sexed IVF embryos resulted in high morphological survival (96.3%) and hatching (84.4%) rates, similar to those fertilized by nonsexed sperm (93.1 and 80.6%, respectively). A 40.9% pregnancy rate was established following the transfer of 3,627 vitrified, sexed embryos into synchronized recipients. This was not different from the rates with nonsexed IVF (41.9%, n = 481), or in vivo-produced (53.1%, n = 192) embryos. Of 458 calves born, 442 (96.5%) were female and 99.6% appeared normal. These technologies (sperm sexing-IVF-vitrification-embryo transfer) provide farmers, as well as the livestock industry, with a valuable option for herd expansion and heifer replacement programs. In summary, calves were produced using embryos fertilized by sex-sorted sperm in vitro and cryopreserved by rapid cooling vitrification.
Key words: sorted sperm, in vitro fertilization, vitrification, embryo transfer

INTRODUCTION

Separation of X- and Y-chromosome-bearing sperm by flow cytometry, and the subsequent application of sex-sorted sperm to fertilize eggs in vivo or in vitro have resulted in offspring of predetermined sex in rabbits, sheep, pigs, horses, cattle, and humans, with accuracy as high as 95% (Johnson, 2000). In vitro fertilization (**IVF**) is a feasible means to increase the fertilization efficiency of sex-sorted sperm (Wheeler et al., 2006). In cattle, approximately 5,000 unsorted sperm (Yang et al., 1993) and as few as 1,500 to 2,250 sexed sperm (Lu and Seidel, 2004) are sufficient to fertilize a bovine oocyte in vitro, whereas 20 million unsorted sperm (Nadir et al., 1993) and as low as 1 million sorted and frozen sperm (Seidel, 1999) are normally used for one AI. Two pioneering studies by Cran et al. (1993, 1995) established pregnancies and produced calves of predictable sex from fresh and frozen IVF embryos fertilized with sorted sperm. Little progress on sexed IVF embryo transfer in cattle has been reported since then. Various problems associated with obtaining satisfactory embryo transfer (**ET**) include the difficulty in obtaining sufficient numbers of sorted sperm with good motility (Schenk et al., 1999), less than optimal IVF and culture systems (Lu and Seidel, 2004; Wilson et al., 2005, 2006), and unsuccessful cryopreservation (Sommerfeld and Niemann, 1999). A few in vitro studies have suggested that IVF efficiency using sorted sperm was affected by sires (Lu and Seidel, 2004), the speed and pressure of the sorting process (Zhang et al., 2003), and transportation of semen (transport duration and temperature) from the bull studs to a sorting facility (Cran et al., 1995). The blastocyst development rate was significantly lower with sorted sperm than with unsorted control sperm (Lu et al., 1999; Wilson et al., 2005, 2006).

The objective of the present study was to systematically improve the efficiency of bovine IVF production using sex-sorted sperm. Effects of sires, time between

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semen collection and sorting, and sperm-sorting pressure on in vitro fertilization rates, and embryonic developmental competence were studied. We utilized a newly developed liquid nitrogen surface vitrification (**LNSV**) method (Du et al., 2004, 2006) with presumptive female IVF Holstein embryos and examined LNSV on subsequent in vitro survival rates of embryos and their in vivo development to birth.

MATERIALS AND METHODS

All chemicals were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted. All embryos were cultured at 39°C in 5% CO_2 and humidified air (unless otherwise specified).

Preparation of Sorted X-Chromosome-Bearing Holstein Sperm

Sorted X-bearing sperm from 9 Holstein bulls (A to I) were produced by flow cytometry using XY sorting methods as described earlier (Schenk et al., 1999) in the sorting facilities at XY Inc. (Fort Collins, CO) and Sexing Technologies Inc. (Navasota, TX). Bulls were selected on their genetic merit and availability, without evaluation of their fertility in vitro. Fresh ejaculates were maintained at 19 ± 1°C for 9 or 14 h before sorting. A total of 200×10^6 sperm were aliquoted into 1.0 mL of HEPES-BSA extender containing 112 μ*M* Hoechst 33342 and stained for 45 min at 34.5°C. One microliter of 5% food coloring (FD&C #40; Sensient Colors, Norfolk, UK) was mixed gently into semen samples for 5 min to stain damaged sperm. Sperm samples were filtered through a 50-μm nylon mesh to remove any debris or clumped sperm before sorting. The variables for flow cytometry were set as follows: sheath pressure at 275.8 to 344.75 kPa, according to the experimental design, 70-μm orifice tip, sort deflection of 1 drop, 150 mW laser power at distinguishing wavelengths of 351 and 364 nm, and average flow rates from 25,000 to 30,000 sperm/s. The X- and Y-chromosome-bearing sperm were sorted into different tubes based on their 3.8% inherent difference in DNA content (van Munster et al., 1999). Approximately 12×10^6 sorted sperm were collected into 2 mL of 20% egg yolk-Tris-A (200 m*M* Tris, 65 m*M* citric acid monohydrate, 56 m*M* fructose), together with 10 mL of sorting medium, and then cooled to 5°C over 90 min. Sorted sperm then received an equal volume of Tris containing 12% glycerol in 2 equal fractions, 15 min apart. The tubes were centrifuged at $850 \times g$ for 20 min at 5°C, the supernatant removed, like pellets pooled, and the sperm concentration adjusted to 10×10^6 sperm/mL with 20% egg yolk Tris containing 6% glycerol. Extended sperm then were packaged in 0.25-mL straws (2.0×10^6 sorted sperm), frozen on racks in a liquid nitrogen vapor phase (–160°C), and stored in liquid nitrogen. The purity of sorted sperm was 90 ± 3% based on re-sort analysis.

Collection and Maturation of Bovine Oocytes In Vitro

Holstein cattle were selected and separated from beef and other dairy breeds at the slaughterhouse 1 d before slaughtering. Cumulus-oocyte-complexes (**COC**) used in this study were collected from ovaries of Holstein cows (Yang et al., 1993). The COC with at least 4 intact layers of cumulus cells were selected and washed 3 times in Dulbecco's PBS (Invitrogen, Grand Island, NY) supplemented with 0.1% polyvinyl alcohol. Oocytes were matured in groups of 25, in 75-μL drops of Medium 199 (Invitrogen) containing Earle's salts, L-glutamine, 2.2 g/L sodium bicarbonate, and 25 m*M* HEPES, supplemented with 7.5% (vol/vol) fetal bovine serum (**FBS**; Hyclone, Logan, UT) and 0.5 μg/mL ovine FSH (National Institute of Diabetes and Digestive and Kidney Disease, Los Angeles, CA), 5.0 μg/mL ovine LH (NIDDK), and 1.0 μg/mL estradiol 17-β. The maturation medium was covered with mineral oil, and COC were cultured for 24 h. The COC with well-expanded cumulus layers at the end of maturation were selected for IVF with sorted X-bearing sperm according to the experimental design.

In Vitro Fertilization with Sorted X-Bearing Sperm

Sorted frozen Holstein X-bearing sperm were used for IVF in Brackett and Oliphant (**BO**) medium (Brackett et al., 1982). Briefly, straws containing semen were thawed for 10 s in a 37°C water bath after 10 s of gentle shaking in air at room temperature. Spermatozoa were washed in 8 mL of BO medium with 3 mg/mL of BSA and 10 m*M* caffeine. The washed sperm pellet was resuspended in BO washing solution at various doses according to the experimental design. Matured COC were rinsed in BO medium containing 6 mg/mL BSA and 10 μg/mL heparin, and 25 allocated per droplet. An appropriate volume of sperm suspension, with a final sperm concentration of 0.15 or 0.3×10^6/mL, was added to each oocyte-containing fertilization droplet according to experimental design. Oocytes and washed sperm were incubated for 6 h in the volume specified for each experiment. Unsorted frozen sperm from the same 4 Holstein bulls were used for the nonsexed sperm fertilization. The procedures for unsorted sperm IVF were the same as described above, except that the final sperm concentration was 0.6×10^6/mL.

In Vitro Culture of Sexed Embryos

Embryos were cultured in CR1aa medium (Rosenkrans et al., 1993). After IVF, cumulus cells were

stripped from presumptive zygotes and washed in CR1aa medium. Presumptive zygotes were randomly allocated to CR1aa culture containing 6 mg/mL of BSA under 5% CO_2, 5% O_2, and 90% N_2, and incubated for 2 d before they were transferred into cumulus cell coculture containing CR1aa medium with 10% (vol/vol) FBS and cultured for a further 5 d. Cleavage status of embryos was evaluated on d 2 (2 to 8 cells), 5 (morula), and 7 (blastocyst) of culture. Embryos were graded according to International Embryo Transfer Society standards (Robertson and Nelson, 1998). Expanding blastocysts with a compact inner cell mass were graded as C1, and selected for cryopreservation. The total number of blastocysts recorded was the sum of grade C1 and C2 embryos (Robertson and Nelson, 1998). The cell number of the C1 grade blastocysts was evaluated by fluorescence microscopy following staining with 10 μg/mL of Hoechst 33342.

PCR Confirmation of Sexed Female Embryos

Samples of the IVF embryos were digested with 20 μL of K-buffer containing 0.1 μg/μL proteinase K (Invitrogen) at 56°C for 45 min, and then held at 95 to100°C for 10 min to inactivate proteinase K. A multiplex amplification of bovine specific autosome and Y-chromosome fragments was performed by one round of PCR (Tominaga and Hamada, 2004). A bovine-specific primer pair was designed to amplify a fragment of 219 bp from a bovine 1.715 satellite DNA as follows: forward: 5′-TGA GGC ATG GAA CTC CGC TT-3′; reverse: 5′-GGT GGT TCC ACA TTC CGT AGG AC-3′. The Y-chromosome fragment (131 bp) was amplified using male-specific primers (forward: 5′-GAT TGT TGA TCC CAC AGA AGG CAA TC-3′; reverse: 5′-GAA CTT TCA AGC AGC TGA GGC ATT TA-3′). One microliter of embryo lysate was used for PCR amplification in a total volume of 25 μL containing 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 2 m*M* $MgCl_2$, 0.2 m*M* dNTP (DNTP100A), 0.4 μ*M* oligonucleotide primers, and 0.5 unit of REDTaq DNA polymerase (Invitrogen). The PCR was initiated with an initial cycle of 95°C for 2 min followed by 30 cycles of 95°C for 30 s, primer annealing at 64°C for 30 s, primer extension at 72°C for 30 s, and a final hold at 72°C for 10 min. Ten microliters of PCR products were analyzed on a 2% agarose gel. One band (219 bp) indicated a female embryo, and 2 bands (219 and 131 bp) indicated a male embryo.

Cryopreservation of Preselected Female Embryos

Embryos were cryopreserved by LNSV (Vitrification and Warming Kit, Evergen Biotechnologies, Inc., Storrs, CT; Du et al., 2004). Briefly, bovine blastocysts were serially incubated in Rinse, Base, and Hold medium for 3 min before vitrification of 4 to 5 embryos in a 2-μL vitrification medium microdroplet, by direct placement onto a thin layer of liquid nitrogen on the solid surface of a metal plate. A group of 30 to 50 vitrified embryos were then transferred into a small freezing vial with a cold fine-tipped forceps, sealed, and stored in the vapor phase of liquid nitrogen (–160°C).

In Vitro Evaluation of Vitrified Female Holstein Embryos

To test the viability of vitrified embryos, frozen embryos were sequentially warmed, rehydrated, and washed in Warm, Rehydrate, and Base media (Evergen Biotechnologies Inc.) at 39°C for 5 min each. Embryo survival was evaluated morphologically under stereomicroscopy when embryos were still alive before fixation. Frozen-thawed embryos were cultured in vitro for 3 d under cumulus coculture containing 10% FBS M199 culture medium, and the survival or hatching rates were recorded at 0, 24, 48, and 72 h. At 72 h of culture, 25 hatched blastocysts from each treatment were stained with Hoechst 33342 and analyzed under fluorescence microscopy for cell counts.

Embryo Transfer of Female Blastocysts

Vitrified presumptive female bovine IVF embryos were transferred into recipients to examine their developmental potential in vivo. Recipient cattle breeds consisted of Chinese Native Yellow Cattle and Holstein cattle on several Chinese farms. Recipients were chosen according to criteria that included: age, health status, breeding history, size and weight, as well as the farm's nutritional management. Recipients were synchronized by a regimen of 2 injections of prostaglandin $F_{2\alpha}$ (Lutalyse, Upjohn Co., Kalamazoo, MI; 25 mg/injection, i.m.) at an interval of 11 d. Corpus luteum (**CL**) regression and estrus usually occurred approximately 48 to 72 h later. The onset of estrus of recipients (d 0) was monitored closely by observing animals that stood when mounted. On d 7 following estrus, recipients were selected by palpation per rectum to verify the presence and the size of the CL. The vitrified microdroplets containing embryos were thawed through a series of steps described above. Blastocysts (1/straw) were loaded into 0.25-mL French straws containing ViGro Holding Plus (AB Technology, Pullman, WA). Straws were maintained at 39°C in a portable incubator for transportation to the farms. A single embryo was deposited nonsurgically into the uterine horn ipsilateral to the ovary with the CL. Pregnancy was determined by palpation per rectum on d 70 after transfer.

Statistical Analyses

The proportions of presumptive zygotes from various treatments reaching cleavage and developing to morula or blastocyst stage within each experiment as well as embryo transfer data were determined and transformed by an arc sine transformation. The transformed data then were analyzed by ANOVA (GLM, SPSS 11.0, Chicago, IL). A 2-way ANOVA with main effects and an interaction was used to analyze the 2×2 experimental designed data in experiments 2 and 3. The mean number of nuclei for postwarmed/vitrified embryos was compared by 1-way ANOVA. A P-value <0.05 was considered significant.

Experimental Designs

Experiment 1: Sorting Influence and Bull Effect on Sexed IVF. The effects of bull or sorting on fertility were determined using 4 bulls (A to D) and compared with fertility of unsorted sperm from the same bulls. The developmental competence of embryos fertilized by sorted sperm from 9 different bulls (A to I) was examined to test the sire's competence for effective sexed IVF. Matured oocytes were randomly assigned to IVF by sorted sperm from the 9 different bulls in a standard 100-μL fertilization droplet. The final concentration was 0.3×10^6 sperm/mL (1,200 sperm per oocyte).

Experiment 2: Minimal Concentration and Number of X-Bearing Sperm for Effective Embryo Development In Vitro. This experiment was designed to determine whether the concentration of sperm could be further decreased for effective fertilization. A 2×2 factorial design was carried out as follows: a fertilization medium volume of 100 vs. 50 μL and final concentrations of sorted X-bearing sperm of 0.3×10^6 vs. 0.15×10^6 sperm/mL from bull A.

Experiment 3: Effect of Sorting Pressure and Length of Storage Time Before Flow Cytometric Sorting on IVF. This experiment was designed (2×2 factorial) to determine: 1) whether IVF was affected by sorting pressure (50 psi, high-speed sorting vs. 40 psi, low-speed sorting), and 2) whether longer periods during the transport of ejaculates before sorting (9 vs. 14 h at 15°C) would adversely affect sperm fertility or embryo development. Long periods before sorting would enable the use of ejaculates from elite bulls that are distant from the few sorting facilities in the nation.

Experiment 4: In Vitro Evaluation and Term Development of Vitrified Female Embryos. This experiment evaluated the in vitro and in vivo developmental competence of vitrified sexed embryos. Embryos were cryopreserved by LNSV, which has proven effective with IVF embryos produced by unsorted sperm (Du et al., 2004). After thawing, the embryos were either cultured in vitro for 3 d or transferred to synchronized recipient animals. Survival rates, hatching rates, and cell counts were recorded for in vitro-produced embryos. The PCR was conducted to confirm the sex of IVF embryos. For ET trials, the vitrified sexed embryos were transferred immediately after warming. The IVF embryos fertilized by unsorted sperm were used as controls for both in vitro and in vivo studies. Commercially purchased in vivo-produced embryos, frozen by conventional slow freezing, were used as controls for the ET trials.

RESULTS

Experiment 1

There was significant variation among the developmental rates of embryos fertilized by unsorted sperm from bulls A, B, C, and D (Table 1). Similar blastocyst rates of IVF embryos were found between sorted and unsorted sperm from bulls A, B, and D ($P > 0.05$), but the blastocyst rate was higher for unsorted than for sexed sperm from bull C (20.7 vs. 0.7%; $P < 0.05$; Table 1).

We further examined the development of embryos produced by sorted sperm from 9 different bulls (A to I). The embryos resulting from sperm from bulls A, E, and F had higher ($P < 0.05$) rates of blastocyst (22.1, 30.4, and 20.1%, respectively) development compared with those of the other 6 bulls (0.7 to 2.1%; Figure 1). The sorted sperm from bulls A, E, and F (3/9 bulls) was highly fertile, and resulting blastocyst development rates were comparable to those of the unsorted sperm of a good IVF bull (bull A, $P > 0.05$). Semen from bull A was selected for use in the remaining in vitro experiments.

Experiment 2

A 2×2 factorial design (fertilization with a sperm concentration at 0.3×10^6 vs. 0.15×10^6/mL and a fertilization drop size of 50 vs. 100 μL; treatments 1 to 4) was conducted to examine the dosage effect of X-sperm fertilization in vitro (Table 2). A higher sperm concentration (0.3×10^6/mL, treatments 1 and 3) for IVF yielded higher ($P < 0.05$) blastocyst development rates (31.0 to 33.2%) than those (11.3 to 12.9%) using a lower sperm concentration (0.15×10^6/mL, treatments 2 and 4), regardless of the size of the fertilization droplet (50 vs. 100 μL). Size of the fertilization droplet did not affect the developmental potential in vitro ($P > 0.05$). The most effective dosage for supporting satisfactory development to the blastocyst stage was 600 sperm per oocyte, at a sperm concentration of 0.3×10^6/mL (treatment 1, 31.0%). Compared with our standard fertilization method (1,200 sperm/oocyte, treatment 3, 33.2%), treatment 1 (600 sperm/oocyte) provided the same embryo yield with half the number of sperm.

Table 1. Bull effect on the development of oocytes fertilized by presexed Holstein sperm

Bull	Treatment[1]	Total oocytes (n)	Embryo development (mean ± SEM)		
			Cleavage, %	Morula, %	Blastocyst, %
A	Sorted	727	69.1 ± 7.4[a]	46.8 ± 7.3[a]	22.1 ± 2.6[a]
	Unsorted	522	60.5 ± 5.1[ab]	32.4 ± 3.8[b]	26.5 ± 4.3[a]
B	Sorted	640	19.6 ± 3.9[c]	3.1 ± 0.6[c]	2.0 ± 0.5[b]
	Unsorted	468	29.6 ± 9.3[cd]	8.5 ± 3.9[c]	7.2 ± 3.4[b]
C	Sorted	720	32.1 ± 1.3[ce]	1.4 ± 0.2[c]	0.7 ± 0.2[b]
	Unsorted	518	78.1 ± 5.9[a]	29.0 ± 6.5[b]	20.7 ± 3.5[a]
D	Sorted	600	46.4 ± 5.4[bdef]	5.0 ± 0.1[c]	1.2 ± 0.4[b]
	Unsorted	524	37.7 ± 5.4[cf]	11.7 ± 1.7[c]	7.3 ± 2.5[b]

[a–f]Values with different superscripts within columns are significantly different ($P < 0.05$). Embryo development to cleavage (2 to 8 cell), morulae, and blastocyst was evaluated on d 2, 5, and 7 post in vitro fertilization, respectively, according to the standards of the International Embryo Transfer Society.

[1]There were 4 replicates for each bull.

Experiment 3

To examine the effect of sperm sorting pressure and the interval between collection and sorting of sperm on IVF, fresh ejaculates from bull A were subjected to the following treatments: 1) sorted at 275.8 kPa, 9 h postejaculation; 2) sorted at 275.8 kPa, 14 h postejaculation; 3) sorted at 344.75 kPa, 9 h postejaculation, and 4) sorted at 344.75 kPa, 14 h postejaculation. The final fertilization concentration of sorted sperm used was 0.3×10^6/mL, and the sperm dose was 600 per oocyte as determined in experiment 2. There was no difference in cleavage or embryonic development to the blastocyst stage among these 4 treatments ($P > 0.05$, Table 3). Neither the increased sorting pressure (344.75 vs. 275.8 kPa), nor the extended period (14 vs. 9 h) postejaculation resulted in adverse effects on blastocyst development rates (Table 3).

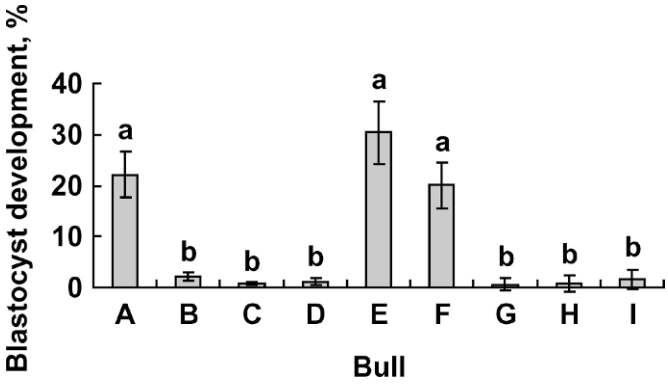

Figure 1. Differential bull effect on in vitro development (embryonic development to blastocyst stage; mean ± SD) of Holstein oocytes fertilized by sorted sperm. Sorted X-bearing sperm from 9 bulls (A to I) were used to examine their fertility via in vitro fertilization (IVF) and culture. Bulls A, E, and F had similar developmental potential during sexed IVF ($P > 0.05$) and significantly higher fertility ($P < 0.05$) than the other bulls.

Experiment 4

Vitrified embryos produced by sorted (n = 164) and unsorted (n = 149, Table 4) sperm from bull A were thawed to examine their survivability and developmental potential in vitro. Vitrified, sexed IVF blastocysts shrunk morphologically and formed a compact cell mass (Figure 2A) immediately after warming; however, they recovered well to expanding blastocysts, morphologically indistinguishable from embryos before vitrification (Figure 2B). No difference was found in the postthawing viability of sexed (96.3%) and control (91.8%) blastocysts (Table 4). Similar hatching rates (84.4 vs. 80.6%) were found in both thawed sexed, and control embryos after 72 h culture postthawing. Twenty-five hatched embryos from each vitrified and warmed sexed and nonsexed group were selected for cell counting and nuclear evaluation. The mean cell number of the vitrified and sexed IVF blastocysts was 538 ± 35 (Table 4), similar to those fertilized with nonsexed sperm (515 ± 33, $P > 0.05$).

One hundred eighteen blastocysts derived from unsorted sperm from bull A and 148 blastocysts derived by sorted sperm from bull A were subjected to PCR sexing. From IVF with unsorted sperm, 54.2% of embryos were female, whereas 95.9% of embryos from IVF with X-sorted sperm were female (Figure 3). This result confirmed the accuracy of the sex sorting of sperm.

Embryo transfer was performed to evaluate the in vivo developmental potential of the sexed, vitrified IVF embryos (Table 4). A total of 3,627 sexed embryos (2,060 by bull A; 1,567 by bull E) were transferred with 1 embryo per recipient. Traditional IVF embryos (n = 481) produced by unsorted sperm and conventional in vivo embryos (n = 192) served as nonsexed control groups. Sexed IVF embryos yielded a pregnancy rate of 40.9% (Table 4), similar to that of nonsexed IVF embryos (41.9%, $P > 0.05$). There was no difference between the pregnancy rates of embryos fertilized by bull A and bull

Table 2. The minimum concentration and number of X-bearing sperm for effective in vitro fertilization (IVF) and embryonic development in vitro[1]

Treatment[2]	Sperm concentration (10^6/mL)[2]	Droplet size (μL)	Ratio of sperm/oocyte[3]	Oocytes (n)	Embryo development (mean ± SEM)		
					Cleavage, %	Morula, %	Blastocyst, %
1	0.3	50	600	285	72.3 ± 1.2[a]	39.4 ± 4.5[a]	31.0 ± 2.9[a]
2	0.15	50	300	258	38.8 ± 1.3[b]	14.1 ± 0.8[b]	11.3 ± 1.0[b]
3	0.3	100	1,200	233	80.8 ± 5.6[a]	45.5 ± 3.1[a]	33.2 ± 2.9[a]
4	0.15	100	600	250	39.1 ± 2.7[b]	14.2 ± 2.4[b]	12.9 ± 2.7[b]

[a,b]Values with different superscripts within columns are significantly different ($P < 0.05$).

[1]There were 3 replicates per treatment. Embryo development to cleavage (2 to 8 cell), morulae, and blastocyst was evaluated on d 2, 5, and 7 post-IVF, respectively, according to the standard of the International Embryo Transfer Society.

[2]Sperm concentration indicates the final concentration of sorted X-bearing sperm used in the droplets according to experimental design. A group of 25 oocytes was arranged into each droplet with a volume of 50 or 100 μL. Depending on the sperm final concentration in each fertilizing droplet, the ratio of sperm to per oocyte was calculated.

E (39.4 and 43.0%, respectively, $P > 0.05$). The pregnancy rates with sexed as well as unsexed IVF embryos were not different from control in vivo embryos (53.1%, n = 192).

Live Holstein calves were produced from the sexed IVF, vitrified, transferred embryos. Presently, the majority of these pregnancies are ongoing. To date, 458 live calves have been born, of which 442 are female (96.5%). Most calves appeared normal and healthy (Figure 2C). In these 458 animals, 2 had physical defects and 1 died within 1 h of birth. From the data collected, 39 abortions were observed from 711 pregnancies at d 70 (5.5%). This was not different from that of nonsexed IVF (5.4%, n = 481) or in vivo-produced (4.8%, n = 192) embryos.

DISCUSSION

In the present study, we achieved 33.2% blastocyst development using X-sorted sperm from a selected bull, which was indistinguishable from results using unsorted control sperm for IVF. Embryonic development to the blastocyst stage was not delayed. Sexed and nonsexed embryos were indistinguishable morphologically with similar cell numbers, similar to the findings of Beyhan et al. (1999). In vitro fertilization is a feasible application for utilizing sorted sperm, based on current sexing technology (Lu et al., 1999; Lu and Seidel, 2004; Wilson et al., 2005). Previous studies with sexed IVF embryos (Cran et al., 1993, 1995; Wilson et al., 2006) indicated that embryos resulting from sexed sperm IVF had an inferior (12 to 27%), and sometimes delayed preimplantation development to blastocyst. The unaffected developmental dynamics of sexed embryos provided evidence that acceptable results can be attained with sorted sperm in an IVF situation. The possible reasons for our improvement include the choice of bull, and improvements to the sorting, fertilization, and culture processes. Brackett and Oliphant fertilization medium, which requires a relatively short sperm-oocyte incubation (6 h) period, was used in our study. In contrast, prolonged sperm-oocyte incubation (18 to 24 h) and a different fertilization system were used (HEPES-chemical defined medium fertilization) by others (Lu and Seidel, 2004).

In the present study we demonstrated that there were both bull and sorting effects that resulted in differential embryo development in sexed IVF and that the bull effect had more influence than the sorting process. The bull effect for IVF is believed due to the difference of sperm

Table 3. The effect of sorting pressure and the time between semen collection and sorting on subsequent development of sexed in vitro fertilized (IVF) embryos

Treatment[1]	Sorting pressure (psi)	Time of sorting (h)[2]	Oocytes (n)	Embryo development (mean ± SEM)		
				Cleavage, %	Morula, %	Blastocyst, %
1	40	9	1191	63.3 ± 6.6[a]	29.7 ± 1.3[a]	23.5 ± 0.9[a]
2	40	14	1207	69.4 ± 7.5[a]	28.1 ± 2.2[a]	25.3 ± 3.0[a]
3	50	9	1433	51.9 ± 9.7[a]	30.3 ± 5.5[a]	20.7 ± 2.5[a]
4	50	14	1288	60.8 ± 1.7[a]	36.5 ± 6.4[a]	23.7 ± 2.1[a]

[a]Values with same superscript within columns are not different ($P > 0.05$).

[1]There were 3 replicates per treatment. Embryo development to cleavage (2 to 8 cell), morula, and blastocyst was evaluated on d 2, 5, and 7 post-IVF, respectively, according to the standard of the International Embryo Transfer Society.

[2]Time of sorting indicates the extended period (h) between semen ejaculation and sperm sorting.

Table 4. In vitro and in vivo developmental competence of sex-sorted in vitro fertilized (IVF) embryos

		Postwarming survivability and development of vitrified embryos, % (mean ± SEM)					Embryo transfer results (mean ± SEM)	
Embryo type[1]	Embryos (n)	2 h	24 h	72 h	Hatched at 72 h	Cell count[2]	No. of recipients[3]	Pregnancies,[4] %
Sorted	164	96.4 ± 1.4[a]	96.3 ± 1.5[a]	96.3 ± 1.5[a]	84.4 ± 3.0[a]	538 ± 35[a]	3,627	40.9 ± 3.6[a]
Nonsorted	149	93.1 ± 1.5[a]	91.8 ± 1.5[a]	91.8 ± 1.9[a]	80.6 ± 3.7[a]	515 ± 33[a]	481	41.9 ± 3.0[a]
In vivo	NA						192	53.1 ± 0.5[a]

[a]Values with same superscript within columns are not different ($P > 0.05$).

[1]Sorted = sexed IVF blastocysts fertilized with sorted X-bearing sperm; Nonsorted = nonsexed IVF blastocysts fertilized with conventional nonsexed semen; In vivo = in vivo embryos cryopreserved by conventional programmed slow freezing protocol; NA, In vivo-derived embryos were not thawed for the survivability test.

[2]Twenty-five hatched IVF blastocysts from sorted and nonsorted vitrification groups were applied for nuclear evaluation by fluorescent microscopy.

[3]Sexed or nonsexed embryos were vitrified on d 7 after IVF. After thawing, only a single embryo derived from either IVF (sexed or nonsexed) or in vivo flushing was transferred nonsurgically into each synchronized recipient.

[4]Pregnancy of recipients was examined by palpation per rectum on d 70 posttransfer.

capacitation for individual bulls during the process of fertilization (Parrish et al., 1986). The 9 bulls used were selected for their genetic merits and availability, but not to their in vitro fertility. After sorting, sperm from bulls A, B, and D (3/4) maintained similar in vitro fertility to sperm that were not sorted; however, the fertility for bull C was significantly decreased. These results indicated that the sorting process affected the in vitro fertility of sorted sperm in a bull specific manner, but it was not a significant factor for all bulls. The bull effect of the sorting process on the in vitro fertility may have reflected recent improvements in sperm sorting technology. In vitro fertilization with conventional sperm from bull C resulted in a significant difference (0.7 to 20.7%, Table 1) in blastocyst development. Likewise, IVF with sorted sperm of 9 bulls (A to I) resulted in significant variation in blastocyst development (Figure 1). Embryos fertilized by X-bearing sperm from bulls A, E, and F (3/9) had significantly higher blastocyst rates than those from 6 other bulls, and would satisfy the need for a large-scale embryo production. Further research is needed to optimize the use of sexed IVF by using different sperm concentration, as suggested by Lu and Seidel (2004).

Our results with different doses of sperm suggested that the sperm concentration (0.15 vs. 0.3×10^6/mL) is more critical than the sperm/oocyte ratio (300, 600, or 1,200 sperm/oocyte), and is dependent upon droplet size (Table 2) in supporting high cleavage and blastocyst rates. The minimum number of X-bearing sperm required to fertilize an oocyte could be reduced by half, from 1,200 to 600, while maintaining a similar and satisfactory blastocyst rate at a sperm concentration of 0.3×10^6/mL if droplet size was 50 vs. 100 μL. This is a >8-fold reduction of our routine IVF with nonsexed sperm (5,000 sperm/egg; Yang et al., 1993). Twenty thousand sorted sperm per oocyte were used in the pioneering work of Cran et al. (1995) and a minimum of 1,500 sexed sperm was used to fertilize 1 egg (Lu and Seidel, 2004).

Sorting pressure (275.8 or 344.75 kPa) and time until sorting (9 or 14 h postejaculation) did not affect the in

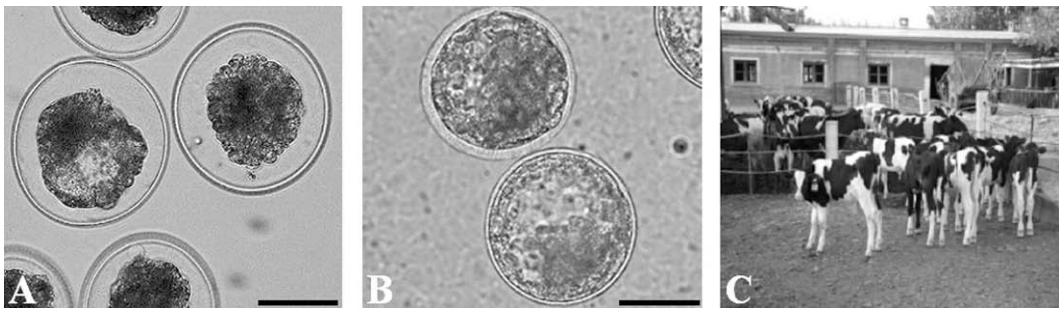

Figure 2. Development of bovine sex-preselected embryos derived by in vitro fertilization with sorted X-bearing sperm and vitrification. Vitrified embryos showed a dramatic shrinkage of embryo mass shortly postwarming (A); but fully reexpanded to the expanding stage before cryopreservation (B) with more than 96% survival and recovery rate after 2 h postwarming. Female Holstein calves were born following embryo transfer of vitrified, sex-preselected embryos (C). Bar represents 80 μm in panels A and B.

Figure 3. Representative gel of PCR sexing of bovine blastocysts fertilized in vitro by sorted X-bearing sperm. Amplification of bovine-specific autosome and Y-chromosome DNA fragments was performed by a multiplex PCR as described in the Materials and Methods. A PCR product of 219 and 131 bp indicated a bovine autosome DNA fragment and specific Y-chromosome DNA amplicon, respectively. The lower 50-bp band = PCR primer dimers; L = 100-bp DNA ladder; N = negative control; M = positive male control; F = positive female control. One band (219 bp) indicated a female embryo, and 2 bands (219 and 131 bp), a male embryo. Lanes 1, 2, 4, 6, and 8 to 15 were identified as positive for females (♀); and lanes 3, 5, and 7, for males (♂). In our PCR experiment, the female ratio was 95.9% (n = 148) in sexed IVF embryos and 54.2% in nonsexed IVF controls (n = 118).

vitro fertility (Table 3). Increasing the sorting pressure could result in increased sorting speeds and throughput. A longer period before sorting makes it possible to acquire fresh ejaculates from top-ranking sires at remote locations and transport them overnight to the sorting facility. This is of practical importance because sires currently need to be located in close proximity to sorting facility, and because there are only very few sorting facilities available.

The LNSV method developed by our group (Du et al., 2004, 2006) resulted in satisfactory performance with nonsexed IVF bovine embryos (Du et al., 2004), in vivo embryos (Lonergan et al., 2005), and cloned embryos (Du et al., 2006). In the present study, 96.3% survival and 84.4% hatching rates were achieved from LNSV of Holstein embryos produced by sorted X-bearing sperm in vitro (Table 4). These results were comparable to their nonsexed IVF counterparts. In addition, the pregnancy/implantation rate of 40.9% was not different from embryos of nonsexed IVF (41.9%) or in vivo (53.1%) controls. This was higher than rates reported previously for nonsexed fresh IVF (Al-Katanani et al., 2002), sexed fresh IVF (Wilson et al., 2005), nonsexed frozen IVF (Al-Katanani et al., 2002), and sexed frozen IVF embryos (Cran et al., 1993, 1995). These results demonstrated that the LNSV vitrification method can cryopreserve sexed IVF embryos with minimal damage to the cells, and that high in vitro and in vivo developmental competence can be attained postthaw.

We report the birth of live calves from vitrified embryos produced from sex-sorted sperm IVF embryos. The ratio of female calves was 96.5%, consistent with our in vitro PCR data (95.9%), and interestingly, higher than the technical specifications provided by XY Inc. (90 ± 3%; Schenk et al., 1999). To our knowledge, this is the first report of live calf production from IVF embryos fertilized by sorted X-bearing sperm and cryopreserved by vitrification. The abortion rate (5.5%) from sexed IVF embryos was not different from that of nonsexed IVF and in vivo embryos. The abortion rate of the sexed IVF embryos was higher than the reported abortion rate of in vivo embryos (3.15%; King et al., 1985), but is much lower than that reported for nonsexed in vitro embryos (13.1%; Hasler, 2000), and is comparable to that achieved by AI with sexed semen (4.5%; Tubman et al., 2004). Less than 1% (2/458) of the calves had physical defects and all others appeared normal. The sperm-sorting processes did not appear to adversely affect the implantation efficiency of the embryos; neither did it seem to cause genetic damage to sorted female embryos. This is consistent with results from other groups in which calves produced by AI with sorted sperm were normal (Tubman et al., 2004).

CONCLUSIONS

In summary, the present study demonstrates an efficient system for the large-scale production, cryopreservation, and transfer of sexed IVF embryos produced by sorted sperm. Both bulls and sorting influenced IVF with sexed sperm. One-third of bulls tested were suitable for sexed IVF embryo production. A minimal number of 600 sorted sperm in 50 μL was sufficient to fertilize an oocyte in vitro, yielding satisfactory developmental rates. Neither increased sorting pressure nor extending the period between collecting the ejaculate and sorting the sperm affected sperm quality and fertility. Over 33% blastocyst development rates from sexed IVF embryos as well as high postwarming survivability of vitrified embryos were achieved. High pregnancy rates (40.9%) were established in a large embryo transfer trial. This, combined with the low abortion rates, predictable sex of offspring, and the birth of healthy calves, demonstrated that a system of sperm sexing, in vitro embryo production, vitrification, and embryo transfer is a feasible and efficient method to produce livestock of the desired sex for the purpose of herd expansion and fast genetic replacement.

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