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In vitro production of bovine embryos using sex-sorted sperm

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Abstract

The objective of this study was to investigate the suitability of sex-sorted sperm for producing viable in vitro embryos for subsequent transfer into recipient cows and heifers on commercial dairy farms. From August 2002 to June 2003, ovaries were collected from 104 producer-nominated Holstein donor cows on seven Wisconsin farms via colpotomy or at slaughter. Oocytes ($N = 3526$) were aspirated from these ovaries, fertilized 22 ± 0.2 h later, and cultured to the morula or blastocyst stage. The fluorescence-activated cell sorting (“Beltsville”) approach was used to produce (primarily) X-bearing sperm from the ejaculates of three young Holstein sires, and 365 transferable embryos were produced. On average, 3.6 ± 0.3 (means \pm S.E.M.) transferable embryos were produced per donor, including 1.4 ± 0.2 (Grade 1), 1.5 ± 0.2 (Grade 2), and 0.7 ± 0.1 (Grade 3) embryos. Number of usable oocytes per donor (33.9 ± 3.3) and percent cleavage (51.1 ± 1.9) were significant predictors of the number of blastocysts that developed. Mean conception rates for the resulting in vitro embryos were $34.2 \pm 1.6\%$ in yearling heifer recipients and $18.2 \pm 0.7\%$ in lactating cow recipients. Additional oocytes ($N = 3312$) from ovaries of anonymous donors (N unknown) collected at a commercial abattoir were fertilized using unsorted sperm, and the percentage of these that developed to blastocyst stage (20.1 ± 2.9) was greater ($P < 0.05$) than the corresponding percentage (12.2 ± 2.3) achieved with sex-sorted sperm using oocytes ($N = 1577$) from the same source. In summary, we inferred that in vitro embryo production may be a promising application of sex-sorted sperm in dairy cattle breeding, but that the biological causes of impaired embryo

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development in vitro and compromised conception rates of transferred embryos should be further investigated.

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1. Introduction

Technologies for sorting bovine sperm into X- and Y-bearing fractions could have a major impact on breeding programs in dairy cattle and other livestock species; several recent papers [1–7] have addressed possibilities, limitations, and potential applications. If cost-effective, procedures that isolate X-bearing sperm could have important economic consequences through enhanced availability and affordability of replacement dairy heifers. Hohenboken [2] hypothesized that the average genetic merit of replacement heifers could be improved through the use of sex-sorted semen. Likewise, Van Vleck et al. [8,9] calculated that producers could benefit financially from sex-sorted sperm, even if it cost twice as much as unsorted sperm. Although sex-sorting of sperm has been the focus of many research projects using a variety of approaches [10–12], this technology has not yet become cost-effective for routine use on commercial farms [1,13]. Presently, the only proven method for sex-sorting of sperm is the fluorescence-activated cell sorting approach of Johnson et al. [14–17]. The so-called “Beltsville Sperm Sexing Technology” is based on a 3.8% difference in DNA content of X- versus Y-bearing bovine sperm. When the DNA of sperm is stained with a fluorescent dye and subjected to flow cytometry, a brighter fluorescence is emitted from the edge of the sperm head compared with the more transparent flat side. Proper sperm orientation can be controlled during flow-cytometric sorting using a beveled nozzle. A charge is applied to each droplet, the droplets are deflected as they pass between two charged plates, and droplets with no charge become waste.

Like sperm-sorting, technology for in vitro production (IVP) of bovine embryos has also encountered many challenges on the path toward widespread commercial application. However, IVP technology may be more useful when combined with sperm-sorting technology [18]. Amann [1] discussed the potential for “tailoring” sperm sorting to the needs and limitations of IVP. Although “niche” applications of these technologies to valuable breeding stock may benefit a few selected individuals, widespread applications on commercial farms will require development of cost-effective breeding programs that can be implemented within the routine management of a dairy or livestock operation. In the case of dairy operations, “low-cost” programs that utilize known (e.g., high genetic merit) or anonymous cull cows as donors may be cost-effective, provided that the timing of the embryo production schemes can be successfully matched with that of the controlled breeding programs on participating farms [19].

The primary objective of this study was to examine the suitability of sex-sorted sperm in IVP of dairy embryos for subsequent transfer into recipient cows or heifers on commercial farms. More specifically, we sought to determine the number of oocytes that could be recovered, the rate of blastocyst development, and the number (and quality) of embryos

that could be produced in a routine in vitro embryo production system utilizing sex-sorted bovine sperm. When possible, we compared these variables with corresponding measurements from unsorted (control) sperm.

2. Materials and methods

Between August 2002 and June 2003, ovaries were recovered from Holstein donors ($N = 104$) on seven commercial dairy farms in Wisconsin. All farms were within 4 h driving time from the IVP laboratory (BOMED, Inc., Madison, WI, USA). Donor animals were selected by these producers from the pool of animals to be culled (in any given month) based on superior genetic merit and/or phenotypic performance. Virtually all donors were “involuntary culls”, i.e., productive, profitable cows whose removal from the herd was forced due to illness, injury, or infertility.

Initially (August–October 2002), ovaries were retrieved surgically on the farm (via colpotomy [20]), such that the farms could continue to milk these cows until the end of lactation. However, because most of the cows were sent to slaughter within a few weeks after surgery, we collected ovaries at the time of slaughter during the remaining months of the study (November 2002–June 2003). On each farm, the schedule for shipping cows to the cooperating commercial abattoir was aligned with the farm’s existing schedule for synchronizing estrus or ovulation. In this manner, the routine reproductive management program of each herd was uninterrupted, except that a portion of the synchronized cows and/or heifers were held an extra 7–8 days as recipients, rather than bred by conventional AI. Following recovery, ovaries from each donor were labeled and stored individually at room temperature (without media) for 1–2 h during the processing of remaining donors, and for 30 min during subsequent transport to the IVP laboratory (additional 30–45 min) and were then transported directly to the IVP laboratory (BOMED, Inc., Madison, WI, USA) for aspiration of oocytes and IVP.

Sex-sorted sperm from three young Holstein sires was used. These sires, which were part of the Accelerated Genetics, Inc., progeny testing program, had previously been housed at XY, Inc. (Ft. Collins, CO, USA) for semen collection and sperm-sorting using the Beltsville Sperm Sexing Technology. Initially, farmers were allowed to choose which sire’s semen would be used for IVP on a given day, but their choice was restricted in the latter months of the study to achieve balanced usage of these sires.

Oocytes from ovaries collected from anonymous donors at a commercial abattoir were used to confirm that the IVP system was functioning properly. All ovaries were transported to the laboratory in an insulated container; transport (from beginning of first ovary collection) took approximately 2–4 h for ovaries that provided control oocytes. Oocytes from follicles between 2 and 8 mm diameter were aspirated (without media) using 18 g hypodermic needles and a vacuum pump (5–6 psi) and were subsequently pooled. After settling for 10–15 min, the pellet was aspirated, washed through a 70 mm tissue filter and decanted into TL-HEPES (AB Technology, Pullman, WA, USA), supplemented with 0.22 mM sodium pyruvate, 50 $\mu\text{g}/\text{mL}$ gentamicin and 3 mg/mL Fraction V BSA [21]. All chemical additives came from Sigma Chemical Co., St. Louis, MO, USA, unless stated otherwise. Oocytes that had three or more layers of compact cumulus cells and evenly

granulated cytoplasm were moved through two more TL-HEPES rinses and placed in maturation medium. Oocytes that were recovered from the ovaries of producer-selected (identified) donors were treated similarly, except that oocytes from each donor were kept separately, and all oocytes that were recovered were placed into maturation medium. Processing of ovaries and oocytes were done at ambient laboratory temperature (22.2 °C).

Oocytes that were recovered were matured for 22 ± 0.2 h in TCM-199 [22,23] with Earles' salts and L-glutamine (Cambrex Bio Science, Inc., Walkersville, MD, USA), supplemented with 0.2 mM sodium pyruvate, 0.025 mg gentamicin sulfate, 10% FCS (also from Cambrex) and 0.01 NIH units of bovine LH and 0.01 NIH units of FSH (Sioux Biochemical, Inc., Sioux Center, IA, USA). Approximately 10 oocytes/50 μ L droplet were incubated under mineral oil. All incubations were performed at 39 °C [24], with 5% CO₂ in air, at high humidity.

Motile frozen-thawed sperm were isolated with a 90–45% Percoll gradient [12], using 0.5 mL of each Percoll concentration, with semen layered on top in a 2.5 mL flat top microcentrifuge tube that was centrifuged for 7 min in a minifuge (Fisher Model 59). One milliliters of TL-HEPES was used to wash sperm (2 min spin), and sperm concentration was determined, adjusted, and added to oocytes that had already been moved to 50 μ L droplets of TL-IVF (Specialty Media, Phillipsburg, NJ, USA) supplemented with pyruvate, gentamicin and 6 mg/mL fatty acid-free BSA. Final concentration of spermatozoa was 1×10^6 mL⁻¹. Hypotaurine, penacillamine and epinephrine were added to induce sperm capacitation [25], along with heparin [26]. Sperm and oocytes were co-incubated for 18 h.

Presumptive zygotes were stripped of cumulus cells, either by vortexing or agitation with a pulled and fire-polished pipette (approximately 150 μ m in diameter). After rinsing through two changes of TL-HEPES, zygotes were placed, 20–30/50 μ L droplet, in SOF (Specialty Media, Phillipsburg, NJ, USA), supplemented with 8 mg/mL fatty acid-free BSA, gentamicin, pyruvate and essential and non-essential amino acids. Late on day 6, FCS (10%, v/v) was added directly to the drops of medium, unless the transfer was to be done on day 8, in which case this step was omitted. Embryos selected for transfer on day 7 or 8 of culture were individually loaded into straws containing TL-HEPES with 10% FCS and placed in an insulated container for transport. Only Grades 1 or 2 morula or blastocyst stage embryos [27] were considered as transferable in our statistical analysis, however, in a few cases ($N = 7$) Grade 3 embryos were transferred.

On day 7 or 8 of culture (day 0 = the day of fertilization in the IVP laboratory), embryos were transported to the cooperating farm (that provided these donors) and transferred (fresh) into recipient Holstein cows ($N = 138$) or Holstein heifers ($N = 76$) into the uterine horn ipsilateral to the corpus luteum. Recipients were either synchronized (e.g., using the Ovsynch program [28]) or were identified based on visible signs of estrus. When the number of available recipients exceeded the number of transferable (Grades 1 and 2) embryos, usable recipients (determined based on palpation results) were chosen at random. When the number of transferable embryos exceeded the number of available recipients, the highest quality (i.e., Grade 1) embryos were transferred first. The remaining embryos were handled in one of three ways, depending on the farmer's preference: (1) remaining embryos were discarded; (2) remaining embryos were transferred into extra recipients on another

farm (two farms in our project had the same slaughter and synchronization schedule); or (3) one or more recipient animals received a bilateral twin transfer. Pregnancy diagnosis (via rectal palpation) was done 35–42 days after breeding.

During the course of this study, the collaborating IVP laboratory also collected ovaries from anonymous (dairy) donor cows (N unknown) at a commercial abattoir. Laboratory controls were generated by fertilizing oocytes ($N = 3312$) from these anonymous donors with unsorted sperm from another Holstein sire, while simultaneously fertilizing oocytes ($N = 1577$) from these anonymous donors with sex-sorted sperm from the three aforementioned young Holstein sires. These data were used to determine the impact of using unsorted versus sex-sorted sperm when generating dairy embryos via IVP. Only laboratory variables (e.g., percent cleavage, percent blastocyst development, etc.) were collected for comparison of IVP embryos from unsorted and sex-sorted semen, because farmers were unwilling to accept IVP embryos that had been generated from unsorted sperm. Because the number of sires was insufficient to compare individual bull differences in embryo development, all comparisons were based on means of bulls providing sex-sorted or unsorted sperm, respectively.

Following an arcsine transformation of percentage data corresponding to cleavage rate and blastocyst development rate, we proceeded with statistical analysis using the following explanatory variables: season, source of oocyte (colpotomy or specific commercial abattoir), number of days of embryo growth, and type of sperm (sex-sorted versus unsorted). Stepwise linear regression analysis with backward elimination was used to determine the significance (or lack thereof) of these explanatory variables on each dependent variable. A linear model (GLM procedure, SAS Institute, Inc., Cary, NC, USA) was used to analyze data regarding the number of blastocysts and the number of transferable embryos (Grades 1 and 2) that developed from each known donor, and quintile groups were created based on of the number of usable oocytes and the percentage that cleaved.

3. Results

Twenty-four laboratory replicates were completed with unsorted sperm; due to limited sperm availability, only 19 replicates were carried out using sex-sorted sperm. As shown in [Table 1](#), the blastocyst development rate from oocytes of anonymous donors fertilized with

Table 1
Means (\pm S.E.M.) for rates of embryo cleavage and blastocyst development from unsorted and sex-sorted sperm using ovaries obtained from anonymous donor cows at a commercial abattoir

	Unsorted spermatozoa	Sex-sorted spermatozoa
Total no. of oocytes	3312	1577
No. of replicates	24	19
No. of oocytes per replicate	138	83
Cleavage rate (%)	67.3 \pm 3.5	65.0 \pm 3.6
Blastocyst development rate (%)	20.1 \pm 2.9 a	12.2 \pm 2.3 b

Within a row, means with different letters differ ($P < 0.05$).

Table 2

Summary statistics corresponding to age, days postpartum, milk yield, oocyte retrieval, and embryo development for producer-nominated (known) donor cows using sex-sorted sperm

Variable	N	Mean (\pm S.E.M.)
Age of cow (years)	104	4.9 \pm 0.2 ^a
Days postpartum at ovary recovery	89	380 \pm 19 ^a
Daily milk yield at last test day (kg)	89	37.7 \pm 1.4
No. of usable oocytes per donor	104	33.9 \pm 3.3 ^a
Embryo cleavage rate (%)	104	51.1 \pm 2.0
No. of morula produced per donor	104	1.0 \pm 0.1
No. of blastocysts produced per donor	104	2.5 \pm 0.3
No. of embryos per donor		
Grade 1	104	1.4 \pm 0.2
Grade 2	104	1.5 \pm 0.2
Grade 3	104	0.7 \pm 0.1
No. of embryos transferred per donor	104	2.6 \pm 0.3 ^a
Conception rate in virgin heifers (%)	76	34.2 \pm 1.6 ^a a
Conception rate in cows (%)	138	18.2 \pm 0.7 ^a b

Within a column, means with different letters differ ($P < 0.05$).

^a As reported by Wilson et al. [29].

sex-sorted or unsorted sperm differed ($P < 0.05$), with significantly more blastocysts produced from the unsorted sperm.

Table 2 shows a detailed summary of data from producer-nominated cows and heifers used as donors in the present study, including age, days postpartum, daily milk yield, oocyte recovery, embryo development, and conception rates. Additional details regarding the milk production and genetic merit of donors and recipients, as well as details regarding the lactation performance and reproductive efficiency of the seven cooperating dairy farms, were provided by Wilson et al. [29]. As noted in Table 2, fewer embryos per donor were actually transferred into recipients (as compared with the number produced) due to shortages of usable recipients for fresh transfers on specific days. Mean conception rates for transferred embryos, as reported previously by Wilson et al. [29], were 34.2 \pm 1.6% for transfers into yearling heifers and 18.2 \pm 0.7% for transfers into lactating cows.

Table 3 shows the number of transferable (Grades 1 and 2) embryos and number of blastocysts produced per producer-nominated (known) donor using sex-sorted sperm, according to quintile groups for the number of usable oocytes and the percentage that cleaved. Quintiles were numbered such that cattle in Quintile 1 had the fewest usable oocytes (or lowest percent cleavage), whereas cattle in Quintile 5 had the most usable oocytes (or highest percent cleavage). Please note that the number of animals per quintile is not identical, due to ties. As expected, donors that produced more usable oocytes or displayed a higher rate of embryo cleavage, tended to produce a greater number of transferable embryos and blastocysts ($P < 0.05$).

Several explanatory variables (i.e. GnRH treatment, season, farm, sire) significantly influenced conception rates, and results of the statistical analysis of on-farm conception rate and calving data corresponding to this study were described in detail by Wilson et al.

Table 3

Means (\pm S.E.M.) for the number of transferable (Grades 1 and 2) embryos and number of blastocysts produced per producer-nominated (known) donor using sex-sorted sperm, according to quintile groups based on the number of usable oocytes and percentage of embryos that cleaved

Quintile	<i>N</i>	No. of usable oocytes	No. of transferable embryos	No. of blastocysts
1	22	7.3 \pm 0.8	0.7 \pm 0.5 a	0.8 \pm 0.5 a
2	20	15.6 \pm 0.5	1.3 \pm 0.5 a	1.1 \pm 0.5 ac
3	21	27.3 \pm 1.1	2.5 \pm 0.5 b	2.3 \pm 0.5 bc
4	21	40.2 \pm 0.7	3.6 \pm 0.5 b	3.5 \pm 0.5 b
5	20	81.6 \pm 11.4	5.9 \pm 0.5 c	4.9 \pm 0.5 bc

Quintile	<i>N</i>	Cleavage (%)	No. of transferable embryos	No. of blastocysts
1	19	18.7 \pm 2.6	1.5 \pm 0.5 d	0.9 \pm 0.5 d
2	24	40.9 \pm 1.1	2.5 \pm 0.5 de	2.0 \pm 0.5 de
3	20	53.3 \pm 0.7	2.9 \pm 0.5 de	2.9 \pm 0.5 ef
4	21	64.0 \pm 0.6	3.4 \pm 0.5 ef	3.3 \pm 0.5 f
5	20	76.4 \pm 1.3	3.8 \pm 0.5 ef	3.4 \pm 0.5 g

Within a column, means with different letters differ ($P < 0.05$).

[29]. As noted by Wilson et al. [29], forty calves were carried full-term; three calves were male, while the remaining 37 were female. One case of “large calf syndrome” was observed (a female calf), and that resulted in the death of both calf and recipient. Wilson et al. [29] also noted that five late-term abortions (>5 months) occurred, although the abortion rate did not appear to be substantially greater than the abortion rate (13%) for AI pregnancies on these farms, and mean gestation length and calving ease score were similar for calves of both sexes.

4. Discussion

The use of flow-cytometrically sorted sperm in IVP systems has been investigated in several previous studies [30–32], and results of the present study generally confirmed those findings. Viable embryos can be cultured to a transferable stage (day 7 or 8) successfully, but a greater number of usable oocytes are needed to develop the same number of transferable embryos when sex-sorted sperm are used.

Given that cleavage rates were similar for IVP with sex-sorted or unsorted sperm, it appears that sex-sorting does not impair the ability of sperm to fertilize oocytes. Rather, it appears that sex-sorting sperm negatively impacts the embryo’s ability to develop normally. Future studies should compare embryo development rates using unsorted and sex-sorted sperm from the same bulls (and perhaps even the same ejaculates), such that any individual sire effects will be negated. However, previous work also suggested that sex-sorted sperm were unable to support blastocyst formation at a rate comparable to that of unsorted sperm [32]. Further investigation into the biological cause(s) of impaired embryo development derived from sex-sorted sperm is needed.

Mean conception rates for embryos transferred into yearling heifers and lactating cows in this study differed ($P < 0.05$), as reported by Wilson et al. [29], and it has been well

documented that yearling heifer fertility is superior to that of lactating cows [33]. However, the conception rate of 34.2% achieved for IVP embryos that were transferred into yearling heifers in this study [29] was far below the expectation for AI in dairy heifers.

In summary, it appeared that female dairy embryos can be produced in a relatively inexpensive manner using sex-sorted sperm and retrieval of ovaries from known or anonymous donor cows at the time of slaughter. Because most culling on commercial dairies is involuntary (i.e., due to injury, illness, or infertility, rather than low milk production), it is unlikely that programs relying on “spent” donors will have a detrimental impact on the genetic potential of participating farms. In practice, collecting ovaries from more donors (perhaps from multiple farms in close geographical proximity) on a given day would help to control variation in the number of transferable embryos produced, and this would allow more accurate prediction of the number of recipient animals needed for fresh transfer of IVP embryos. Research should continue to focus on methods to improve embryo development and conception rates using sex-sorted sperm. In addition, alternative models of commercial implementation that capitalize on the benefits of an altered sex ratio while minimizing the negative aspects of sperm-sorting (e.g., high price, reduced fertility) should be investigated.

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