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Fertilization rate and embryo quality in superovulated Holstein heifers artificially inseminated with X-sorted or unsorted sperm

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Abstract

Although studies have demonstrated reasonable pregnancy rates in single-ovulating heifers inseminated with sexed sperm, it is not yet clear if sexed sperm can be successfully used in superovulated females. Therefore, the objective of this trial was to determine fertilization rate and embryo quality in superovulated heifers inseminated with frozen-thawed X-chromosome bearing sperm at 2 different dosages versus unsorted sperm. Nulliparous Holstein heifers (n = 23; 12 to 16 mo) were superovulated with FSH and observed for estrus twice a day. Following detection of estrus, bilateral deep uterine horn AI was performed once (1X) or twice (2X) in each heifer, according to the treatments: S20-1X: AI with 20 million X-sorted sperm 12 h after the onset of estrus; S10-2X: AI with 10 million X-sorted sperm 12 and 24 h after the onset of estrus; and U10-2X: AI with 10 million unsorted sperm 12 and 24 h after the onset of estrus. Embryos/ova were collected 7 d after estrus and evaluated. Ten d later, heifers were resynchronized for a second superovulatory treatment and assigned to a second treatment group. Data were compared using mixed effects models. When sexed sperm were used, number and percentage of fertilized and viable embryos recovered per flush was similar between the S20-1X and S10-2X group (63.5% and 61.9%), but lower ($P < 0.01$) than in the U10-2X group (90.9%). In addition, heifers bred with X-sorted sperm had an increase ($P < 0.05$) in the percentage of degenerate embryos when only fertilized structures were included in the analysis (S20-1X = 58.6%, S10-2X = 53.1%, U10-2X = 24.2%). In conclusion, it seems likely that the sorting process may have caused damage in the sperm that compromised fertilization as well as subsequent embryonic development in superovulated heifers.

Keywords: sexed sperm, superovulation, embryo, heifers.

Introduction

Multiple ovulation and embryo transfer (MOET) programs have been designed to produce genetically superior offspring by superovulating selected dams and breeding these dams to superior sires by AI (Merton *et al*., 2003). One way to increase the production of these genetically superior offspring is to increase the efficiency of the superovulation/embryo transfer techniques. However, in spite of recent advances in manipulation and control of follicular waves that have improved consistency of superovulation, there remains substantial variability and many times suboptimal results with this technique (Mapletoft *et al*., 2002). One potential method to increase efficiency of these procedures is to generate viable embryos of the desired sex. For example, in the dairy industry female embryos and calves are of much greater value than male embryos/calves. Sexing of the embryos after flushing allows selection of embryos of the desired sex but does not increase the number of embryos of the desired sex that are generated. One potential method to increase the production of sexed viable embryos is to breed superovulated animals with semen that has been previously sorted to enrich the population of X-bearing or Y-bearing spermatozoa.

The use of sexed sperm to improve cattle production systems has been extensively discussed (Hohenboken, 1999; Seidel Jr *et al*., 1999). Previous studies have demonstrated reasonable pregnancy rates

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in single-ovulating heifers inseminated with sexed sperm (Seidel Jr *et al*., 1999). However, we have been unable to find previous research reports that have examined the use of sexed sperm in superovulated cattle. Therefore, the objective of this trial was to determine fertilization rate and embryo quality in superovulated heifers inseminated with frozen-thawed X-chromosome bearing sperm at 2 different dosages versus unsorted sperm from the same bulls.

Materials and Methods

Intravaginal progesterone (P_4) implants (Eazi-Breed CIDR containing 1.9 g of P_4) were from InterAg Company (Hamilton, New Zealand). Prostaglandin $F_{2\alpha}$ (ProstaMate) was from Phoenix Pharmaceutical Inc. (St. Joseph, MO, USA). The GnRH (Cystorelin) was from Merial Limited (Iselin, NJ, USA). Sesame oil and β-estradiol 3-benzoate (EB) were from Sigma Chemical Co. (St. Louis, MO, USA). Benzyl alcohol was from EM Science (Cherry Hill, NJ, USA). The FSH (Folltropin-V; equivalent to 400 mg of NIH-FSH-P1) was from Vetrepharm Canada Inc. (Belleville, Ontario, Canada). Kamars were from Kamar Inc. (Steamboat Springs, CO, USA).

Superovulation, insemination, embryo recovery and evaluation

Nulliparous Holstein heifers (n = 23; 12 to 16 months old) were housed indoors at the Livestock Laboratory at the University of Wisconsin-Madison, USA in a concrete area containing headlocks during the spring of 2002. Heifers had free access to water and were fed twice a day a ration consisting of alfalfa silage and concentrate based on wheat, soybean hulls, and corn grain. The heifers were synchronized with a protocol that involved the use of a CIDR for 9 d (Day 0 to 9), combined with an injection of EB (2.5 mg, im, diluted in sesame oil and benzyl alcohol) on Day 1, and a PGF2α (25 mg, im.) injection 12 h before CIDR removal. Heifers were treated from Day 6 to Day 9 with decreasing doses of FSH (Folltropin-V; 3, 3, 2, 2, 1.5, 1.5, 1, 1 ml, using a total dose equivalent to 300 mg of NIH-FSH-P1, im) every 12 h. After CIDR removal, heifers were visually observed for standing estrus at 7 a.m. and 7 p.m. for 20 min and fitted with a pressure-activated heat mount detector (Kamar) that was checked twice daily. Estrus was designated by either visual observation of standing estrus or the presence of an activated Kamar. Following detection of estrus, heifers were bred by bilateral deep uterine horn AI according to assigned treatment groups. The treatment groups were: S20-1X = AI with 20 million X-sorted sperm 12 h after the onset of estrus; S10-2X = AI with 10 million X-sorted sperm 12 and 24 h after the onset of estrus; U10-2X = AI with 10 million unsorted sperm 12 and 24 h after the onset of estrus. One technician did all inseminations. As discussed below, all heifers went through 2 separate superovulations and were assigned to a different AI treatment in each superovulation (each heifer is represented once in each of 2 of the treatments).

Three bulls were chosen for use in this study (Select Sires, Inc., Plain City, OH, USA). Semen was collected from each bull and X and Y bearing spermatozoa were separated using flow cytometry and processed similarly to previous description (Schenk et al., 1999). The only exception to those methods in Schenk et al. (1999) is that the sperm were sorted using 40 psi rather than 50 psi. Semen was stored in 0.25 ml straws at 10 million spermatozoa/straw (S10-2X and U10-2X) or 20 million spermatozoa/straw (S20-1X). Each bull had semen in all treatment groups with heifers being randomly assigned to bull as well as AI treatment group.

Embryos/ova were collected nonsurgically 7 d after detection of estrus. All uterine flushings were performed by the same experienced technician. Embryos/ova were collected, counted, and evaluated for fertilization. Embryo quality was graded according to the IETS guidelines (Robertson and Nelson, 1998). Grade 1 and 2 embryos were defined as viable, and grade 3 and 4 were considered degenerate. Unfertilized ova were designated when there was no sign of cleavage. Grade 1 embryos were frozen in liquid nitrogen. Degenerate embryos and unfertilized ova were evaluated for number of accessory sperm (AS) as previously described (Sartori et al., 2002). Number of ovulations per ovary per heifer was estimated using an ultrasound scanner (Aloka 500-V; Corometrics Medical Systems Inc., Wallingford, CT, USA) equipped with a 7.5 MHz linear-array transducer. The number of follicles ≥ 8.5 mm at the time of standing estrus was determined and ovulations were designated by disappearance of these follicles 48 h later. The mean diameter of 8.5 mm was chosen because it was the smallest size of superstimulated follicles that ovulated after estrus in the present experiment. In addition, number of corpora lutea (CL) was confirmed by ultrasound on the day of embryo collection.

On the day of embryo collection, all heifers received 2 injections of PGF2α (25 mg, im) 12 h apart. Ten d later, heifers were resynchronized for a second superovulation treatment and were assigned to a second treatment group. Therefore, each heifer was included in 2 of the available 3 treatments. Any embryo collections that had only 1 or no embryos/ova recovered were not included in the analysis (2 in S10-2X, 3 in S20-1X, and 2 in U10-2X), except for the evaluation of recovery rate of embryos/ova per CL per flush.

Statistical analyses

The binomial distribution (macro GLIMMIX of SAS) was assumed for variables related to the presence of AS. The Poisson distribution (macro GLIMMIX of SAS) was assumed for the count variables, such as CL number, and embryos/ova per flush.The continuous variables, such as percentage of embryos and/or ova per flush were evaluated using a mixed effects model (procedure MIXED of SAS; Littell *et al.*, 1996). Besides the treatments, the models also included the date of data collection, bull, and heifer, which were treated as random effects.

Results

There was no difference among treatment groups for the number of ovulations (CL number) after the superstimulatory treatment (Tab.1). There was, however, a difference in the number of embryos/ova recovered per flush between groups. The S20-1X group had fewer embryos/ova recovered than the U10-2X group (Tab.1). Percentage of embryos/ova recovered per CL per flush was lower ($P < 0.05$) for the S20-1X group than for the U10-2X group either when all data were analyzed (S10-2X = 39.1% vs. U10-2X = 59.3%) or when only data from flushes with ≥ 2 embryos/ova recovered were compared (S10-2X = 44.1% vs. U10-2X = 66.6%). Recovery rate per CL for the S10-2X group was intermediate (~ 50%) and did not differ ($P > 0.10$) from the other 2 groups. When sexed sperm was used, there was about a 60% fertilization rate that did not differ between the S20-1X and S10-2X groups (Tab.1). However, the fertilization rate was greater for the animals bred with unsorted sperm (U10-2X = 90.9%). Similarly, the number of fertilized embryos per flush was also greater in the unsorted group than sorted sperm groups (Tab.1). The percentage of viable embryos per flush was not different for the 2 groups of X-sorted sperm but was much lower than for the unsorted sperm (Tab.1). Although the number of degenerate embryos per flush did not differ between groups, the percentage of degenerate embryos per flush tended ($P < 0.10$) to be greater for the 2 groups with sorted sperm than the group with unsorted sperm (Tab.1). There was an increase in the percentage of degenerate embryos per fertilized embryo in the groups bred with X-sorted sperm (~ 55%) compared to the group bred with unsorted sperm (24%).

In general, the minority (23.8%; 43/181) of the degenerate embryos and unfertilized ova had 1 or more AS trapped in the zona pellucida. Moreover, among the embryos/ova with AS, 81.4% (35/43) presented only ≤ 3 AS. Among treatment groups, heifers inseminated only once with sexed sperm (S20-1X) after estrus presented fewer embryos/ova with AS (Tab.2).

Table 1. Results (mean ± SEM) from superovulated heifers inseminated with X-sorted sperm (S20-1X: 20 million, or S10-2X: 10 million sperm/dose), or unsorted sperm (U10-2X: 10 million sperm/dose).

	S20-1X (n = 12)	S10-2X (n = 13)	U10-2X (n = 14)
CL number at flushing	15.3 ± 1.7	18.1 ± 3.4	14.1 ± 1.5
Embryos/ova per flush	6.8 ± 1.6[a]	8.9 ± 1.8	9.9 ± 1.9[b]
Fertilized per flush	3.8 ± 0.8[a]	4.9 ± 0.9[a]	8.7 ± 1.7[b]
% Fertilized per flush	63.5 ± 9.2[a]	61.9 ± 6.3[a]	90.9 ± 4.0[b]
Viable per flush	1.9 ± 0.7[a]	2.3 ± 0.6[a]	6.3 ± 1.2[b]
% Viable per flush	24.3 ± 8.5[a]	30.8 ± 7.7[a]	71.3 ± 7.3[b]
Degenerate embryos per flush	1.8 ± 0.4	2.6 ± 0.6	2.4 ± 0.9
% Degenerate per flush	39.2 ± 10.2	31.1 ± 5.6	19.6 ± 4.8
% Degenerate of fertilized	58.6 ± 11.8[a]	53.1 ± 9.8[a]	24.2 ± 6.4[b]

[a,b]Different within each row; $P < 0.05$.

Table 2. Percentage of degenerate embryos and/or unfertilized ova with accessory sperm in the zona pellucida. Embryos/ova were recovered from superovulated heifers inseminated with X-sorted sperm (S20-1X: 20 million, or S10-2X: 10 million sperm/dose), or unsorted sperm (U10-2X: 10 million sperm/dose).

	S20-1X (n = 12 heifers)	S10-2X (n = 12 heifers)	U10-2X (n = 10 heifers)
Degenerate embryos	28.6% (6/21)	30.3% (10/33)	44.0% (11/25)
Unfertilized ova	5.9% (2/34)	24.5% (13/53)	6.7% (1/15)
Total	14.5% (8/55)[a]	26.7% (23/86)	30.0% (12/40)[b]

[a,b]Different within each row; $P < 0.05$.

Discussion

With the desire of controlling the sex of the offspring, for decades researchers have investigated different techniques to sexing sperm, but as mentioned by Seidel Jr (2003), to date only one procedure has proven efficacious in any practical sense. That procedure utilizes flow cytometry to separate X- and Y-bearing spermatozoa based on the DNA content of individual sperm as described by Johnson (2000). This technique has been found to have excellent accuracy and results in sorted populations of viable sexed sperm. Seidel Jr *et al.* (1999) working with single-ovulating beef heifers reported that pregnancy rates with sexed, frozen sperm were within 90% of unsexed, frozen controls that had 7 to 20 times more sperm/insemination dose.

Based on the encouraging results obtained with sexed sperm in single-ovulating heifers, we evaluated the use of frozen-thawed X-chromosome bearing sperm in superovulated heifers. In spite of using high doses of sperm (10 to 20 million) per AI in the present experiment, as compared with the experiments in single-ovulating heifers (1 to 3 million; Seidel Jr *et al.*, 1999), the fertilization rate in the females bred with sexed sperm was much lower than the control group. Besides the low fertilization rates with sexed sperm observed in this study, there was also an increase in the percentage of degenerate embryos for the sexed sperm groups when only fertilized structures were analyzed. The combination of these factors culminated in a 3 times lower number of viable embryos per flush in the groups in which sexed sperm was used. From these results it appears that the procedure used in this study for sexing the sperm caused damage to the sperm that compromised fertilization rate (30% decrease). These unexpected negative results observed with the sexed sperm in superovulated heifers may be related to the peculiar reproductive physiology and endocrinology of superovulated cattle (Kafi and McGowan, 1997). Although high fertilization rates are common in single-ovulating beef and dairy cows and heifers (Ahmad *et al.*, 1995; Dunne *et al.*, 2000; Dalton *et al.*, 2001; Sartori *et al.*, 2002), data from superovulated cattle indicate lower fertilization rates (Elsden *et al.*, 1976; Hawk and Tanabe, 1986; Hawk, 1988; Saacke *et al.*, 1998; Dalton *et al.*, 2000; Sartori *et al.*, 2003). As discussed by Kafi and McGowan (1997), the lower fertilization rate in superovulated cattle may be due to disturbances in sperm and ova transport and suboptimal oocyte quality. Therefore, a potentially suboptimal quality of the sorted sperm associated with suboptimal conditions provided by the superovulated female, resulted in lower fertilization rates when compared to the unsorted control group.

Unexpectedly, there was also an increase in the percentage of fertilized embryos that were degenerate (53 to 59% for the sorted sperm vs. 24% for unsorted). This result suggests that in addition to sperm damage that decreases fertilization efficiency, there may be extra damage to the sperm that results in compromised post-fertilization embryo development. This result was not expected and will need to be confirmed in subsequent investigations.

The significant differences in recovery rate per CL and number of embryos/ova recovered per flush between the group that received only 1 AI with sorted sperm after estrus (S20-1X) compared with the group that received 2 AI with unsorted sperm (U10-2X) are difficult to explain. One speculative reason could be that because the S20-1X group had lower fertilization rate than the U10-2X group, less embryos/ova were transported from the oviduct to the uterus. It is not known if, in the bovine species, the transport of unfertilized ova from the oviduct to the uterus is similar to the transport of embryos. It may be possible that more unfertilized structures (and perhaps, more degenerate embryos) were retained in the oviduct than healthy embryos, as observed in mares in much greater proportions (Betteridge and Mitchell 1974; Betteridge *et al.*, 1979; Ginther, 1992). If this is the case, the results presented in Tab. 1 for fertilization rate of the groups in which sexed sperm was used may be overestimated. Following the same idea, the results presented in Tab. 1 for percentage of degenerate embryos may be underestimated in the sexed sperm groups. Despite the low fertilization rate, similar to the S20-1X group, the S10-2X group presented intermediate results for recovery rate per CL or number of embryos/ova recovered per flush. These intermediate results are difficult to explain, but may be related to the fact that this group was inseminated at 12 and 24 h after the onset of estrus, as the U10-2X group was.

Studies (Nadir *et al.*, 1993; Saacke *et al.*, 1998; Sartori *et al.*, 2002) that evaluated number of AS trapped in the zona pellucida of embryos/ova of single-ovulating cows and heifers reported that the great majority of embryos had at least 1 AS. For example, Sartori *et al.* (2002) reported averages or median numbers of AS in embryos from dairy cows or heifers ranging from 18 to 42. Moreover, 80% of the unfertilized ova from cows had at least 1 AS, and on average, ova had 18 AS in the zona pellucida. When evaluating number of AS in superovulated cows, however, studies have shown much lower percentages (5 to 41%) of embryos with AS in the zona pellucida (Hawk and Tanabe, 1986; Hawk, 1988; Saacke *et al.*, 1998; Dalton *et al.*, 2000). In the study by Saacke *et al.* (1998) only 10% of the fertilized ova from superovulated cows had AS, in contrast to 61% of the embryos from single-ovulating cows having AS. The very low percentage of embryos/ova with AS in the present study is in agreement with other studies. However, only degenerate embryos and unfertilized ova were evaluated for AS in the present study and therefore it is difficult to compare to previous studies with all embryos/ova evaluated for AS.

The finding that heifers in the S20-1X group

had fewer embryos/ova with AS than the U10-2X group may be a combination of suboptimal timing of AI and suboptimal sperm quality. In the S20-1X group, 20 million sorted sperm were inseminated once at 12 h after the onset of estrus, whereas in the U10-2X or S10-2X groups 20 million sperm were also used for AI, but divided into 2 doses of 10 million inseminated at 12 and 24 h after the onset of estrus. In contrast to single-ovulating cattle, the best time for AI in superovulated cattle seems to be 24 and not 12 h after the onset of estrus (Dalton et al., 2000). This may help explain why there was a difference in AS between S20-1X and U10-2X heifers, but not between S10-2X and U10-2X heifers.

In conclusion, the process of sorting X-bearing sperm appears to result in some damage to the sperm that compromised fertilization rate and subsequent embryonic development in superovulated Holstein heifers.

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