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Fertilization rate and embryo production of superovulated dairy cows after insemination with non-sorted and sex-sorted semen

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Abstract

The aim of this study was to evaluate the fertilization rate of cows that were superovulated and artificially inseminated with sex-sorted semen. Cows were treated with an intravaginal progesterone device plus estradiol benzoate (day 0). Superstimulation treatments began four days after with eight applications of FSH at 12 h intervals. D-Cloprostenol was administered on day 6. Progesterone device was removed on day 7, and LH was administered on day 8. The treatments were divided as follows: NonSx, two AI with non-sorted semen were conducted 12 and 24 h after LH; Sx12&24, two AI with sex-sorted semen were conducted 12 and 24 h after LH; and Sx24&36, two AI with sex-sorted semen were conducted 24 and 36 h after LH. Embryos were recovered on day 16 and were evaluated and classified. Percentage of fertilized embryos tended to be greater for the non-sorted semen than the sex-sorted semen. The number of unfertilized oocytes was smaller when the non-sorted semen was used relative to the sex-sorted semen. There was no difference between the treatments that used sexed semen. In conclusion, the use of sex-sorted semen in superovulated dairy cows results in greater numbers of unfertilized oocytes than non-sorted. However, when only sorted semen is used AI should be performed 24 and 36 h after LH.

Keyword: Brown swiss, sexed, sexing, superovulation.

Introduction

Sperm that is separated according to the sex chromosomes (X or Y) can prevent sex-linked genetic diseases, save endangered animals and increase productive efficiency (Seidel and Johnson, 1999). In cattle, females are essential for calf and dairy production, while males are important for beef production. Thus, sex-sorted semen is of great interest due to its handling and economic advantages (Hamano, 2007).

Despite considerable advances in the sex-sorted semen process, the fertilization rates are not the same as those obtained from non-sorted semen (Garner, 2006; Seidel and Schenk, 2008; Karakaya *et al*., 2014). Several studies have reported the use of different strategies for increasing the results of artificial insemination (AI) using sex-sorted semen in superovulated donors. These strategies include using different sperm concentrations (Sartori *et al*., 2004; Schenk *et al*., 2006), using fresh sex-sorted semen (Hayakawa *et al*., 2009), using a higher number of AIs (Peippo *et al*., 2009; Larson *et al*., 2010) and using AI at different times (Soares *et al*., 2011). The use of sex-sorted semen in superovulated cattle is promising when considering the limitations of *in vitro* embryo production related to cryopreservation (Viana and Camargo, 2007).

The use of timed AI (TAI) requires hormonal treatments that ensure adequate control of follicular development and luteolysis for ovulation synchronization. These aspects have been controlled in superovulation (SOV) protocols in which the use of GnRH or LH for ovulation induction has been widely used (Nogueira *et al*., 2002; Chesta *et al*., 2005; Bo and Mapletoft, 2014), reducing the interval between the first and last ovulations (Chesta *et al*., 2005). Seventy-five percent of the ovulation in superovulated *Bos taurus* cows occurred between 24 and 36 h after administration of 25 mg of LH (Martins, 2007).

Therefore, the aim of this study was to evaluate the fertilization rate and embryo production in superovulated *Bos taurus* cows that were inseminated in fixed time with sex-sorted and non-sorted semen. The hypothesis of this study was that TAI in superovulated cows should be performed as close as possible to the time at which a greater number of ovulation occurs to increase the fertilization rate with sex-sorted semen.

Materials and Methods

The Animal Research Ethics Committee of the Federal Rural University of Pernambuco approved all of the procedures involving animals in this study.

Location and animals

This study was conducted on a dairy farm in Pernambuco State in Northeast Brazil from January to October 2010. During this experiment, the average temperature was 22.2°C, which varied from 16.8 to 32.3°C, and the humidity varied from 39 to 100%. Nine multiparous and non-lactating Brown Swiss dairy cows were selected, with an average body score condition of 3.4 ± 0.2 [1-5, in a 1 (emaciated) to 5 (obese) scale

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(Wildman *et al.*, 1982)], at 4-8 years old and an average weight of 617.1 ± 56.3 kg. Only healthy animals were selected for the experiment. The diet of the cows consisted of pasture (*Brachiaria decumbens*), ground corn and soybean meal, minerals, and vitamins. The first SOV began after two months of supplying the diet.

Superovulation and artificial insemination

All cows received a progesterone releasing intravaginal device (P4; PRIMER®, Tecnopec, São Paulo, Brazil) with 3.0 mg of estradiol benzoate (EB; RIC-BE®, Tecnopec, São Paulo, Brazil) at an unknown stage in their estrous cycle (day 0). Between days 4 (day 4) and 7 (day 7), the cows were superovulated with 200 mg i.m. of pFSH (Folltropin-V®, Bioniche Animal Health, Ontario, Canada) in decreasing doses (40, 40, 30, 30, 20, 20, 10, and 10 mg) twice each day. On day 6 (day 6) and during the fifth and sixth doses of FSH treatments, all of the animals received 0.150 mg i.m. of d-Cloprostenol (Prolise®, ARSA S.R.L., Buenos Aires, Argentina). The P4 devices were removed during the last day of FSH treatment and 24 h after (day 8) all of the animals received 25 mg i.m. of pLH (Lutropin®, Bioniche Animal Health, Ontario, Canada). The experiment was conducted in a latin-square design, where each cow was superovulated three times for 27 superovulations. The minimum interval between the superovulations was two months.

According to semen type (sex-sorted or non-sorted) and the time of the AI, the cows were randomly separated into three groups, NonSx (n = 9) = two AIs with non-sorted semen (20.0 x 10^6 sperm/dose) conducted 12 and 24 h after administration of the LH (control group), Sx12&24 (n = 9) = two AIs with sex-sorted semen (2.0 x 10^6 sperm/dose) conducted 12 and 24 h after administration of the LH and Sx24&36 (n = 9) = two AIs with sex-sorted semen (2.0 x 10^6 sperm/dose) conducted 24 and 36 h after administration of the LH. Figure 1 shows the protocols used for estrus synchronization and superstimulation in the *Bos taurus* cows that were inseminated two times according to their experimental group. The semen (sex-sorted and non-sorted) used in the experiment was obtained from the same ejaculate of one bull in the semen Central, which was previously evaluated and approved according to the minimum standard requirement.

The embryos were collected by an experienced veterinarian using standard nonsurgical uterine flushing techniques seven days after the AI. The flushing was performed using 1000 mL of Dulbecco's phosphate-buffered saline solution (PBS, Nutricell Nutrientes Celulares, Campinas, São Paulo, Brazil) supplemented with 1% fetal calf serum (Nutricell Nutrientes Celulares). The recovered ova/embryos were separated and evaluated according to the IETS manual (Robertson and Nelson, 1998) as grade 1 (excellent or good), 2 (fair), or 3 (poor). Grade 1 and 2 embryos were considered transferable. After the embryo collection, the numbers of corpus luteum were observed using the transrectal ultrasound technique (Falcon Vet® 100, Pie Medical, Maastricht, Holanda) with a 6.0 MHz linear transducer.

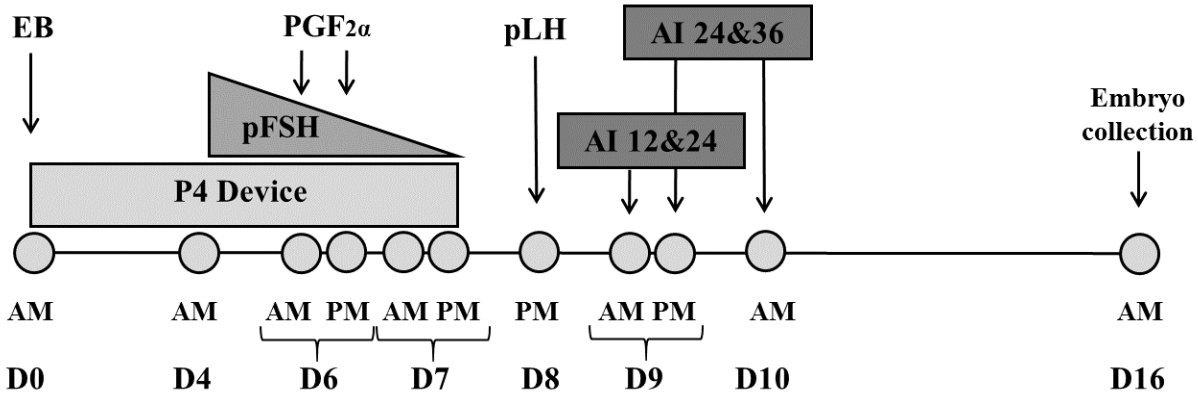

Figure 1. Treatment protocols for synchronization and superstimulation in *Bos taurus* cows that were inseminated two times according to their experimental group. AM - 6:00 AM; PM - 6:00 PM; P4 - Progesterone; EB - Estradiol benzoate at a dose of 3 mg; PGF2α - Prostaglandin F2α; pFSH was administered between day 4 and day 7 twice each day; NonSx and Sx12&24 – AI day 9 morning and afternoon; Sx24&36 - AI day 9 in the afternoon and day 10 in the morning.

Statistical analysis

The data were analyzed using the Glimmix procedure in SAS (System for windows, Version 9.3; SAS Institute Inc., Cary, NC, USA). The model included the fixed effects of treatment and the random effect of latin-square. Orthogonal comparisons were used to determine the effects of semen type (NonSx *vs.* Sx12&24 + Sx24&36) and the effects of AI time (Sx12&24 *vs.* Sx24&36). Differences of $P \leq 0.05$ were considered significant and differences of $0.05 < P \leq 0.10$ were considered tendencies. Data are shown as the least square mean ± standard error. The cows that did not respond to superovulation or did not have embryo/ova recovered were excluded from the statistical analyses.

Results

Of the 27 performed superovulations, five cows did not produce compatible ova/embryos following the ovarian superstimulatory response, one from the NonSx group and four from the Sx24&36

group. From the five superovulations that did not have a stimulatory response, two were from a donor that only showed responses in the Sx12&24 group.

There were no differences in the numbers of corpus luteum and the recovered structures number/flushing. In addition, the proportion of fertilized embryos was also similar among the treatments. However, the proportion of fertilized tended (P = 0.09) to increase in the non-sorted semen relative to the sorted semen. No effects of semen type on the transferable embryo were observed, but the Sx24&36 group generally (P = 0.09) had more transferable embryos than the Sx12&24 group. The number of degenerated embryos per flush tended (P = 0.08) to be greater for the non-sorted semen than sex-sorted semen. A treatment effect (P = 0.05) was observed for the proportion of degenerated embryos, where the NonSx had greater numbers compared to the Sx12&24 and Sx24&36 groups. The number of unfertilized oocytes per flush was smaller (P = 0.05) when the non-sorted semen was used rather than the sorted semen. The proportion of cows with unfertilized ova was smaller for the NonSx group than for the Sx12&24 and Sx24&36 groups (Table 1).

No difference was observed when evaluating the proportions of cows with more than 50% of fertilized embryos per flush (Table 1).

Table 1. Results (least square mean ± SE) from superovulated cows inseminated with nonsorted and sex-sorted semensorted sperm.

	Treatments[1]			P-value[2]		
	NonSx (n = 8)	Sx12&24 (n = 9)	Sx24&36 (n = 5)	TRT	Semen type	AI Time
Corpus luteum number per cows[3] (n)	11.7 ± 1.27	11.2 ± 1.20	11.7 ± 1.59	0.94	0.85	0.81
Embryo/oocyte per flush (n)	8.4 ± 2.53	10.0 ± 2.40	9.4 ± 3.16	0.87	0.65	0.87
Fertilized per flush(n)	5.4 ± 1.90	1.0 ± 1.79	3.8 ± 2.40	0.28	0.24	0.37
Fertilized per flush (%)	47.3 ± 12.00	15.1 ± 11.27	22.6 ± 15.12	0.18	0.09	0.70
Transferable embryo per flush (n)	0.3 ± 0.72	0.3 ± 0.68	2.4 ± 0.91	0.17	0.25	0.09
Transferable embryo per flush (%)	9.8 ± 4.50	4.7 ± 4.22	16.0 ± 5.72	0.32	0.93	0.15
Degenerate embryo per flush (n)	4.6 ± 1.48	0.7 ± 1.40	1.4 ± 1.88	0.18	0.08	0.76
Degenerate embryo per flush (%)	39.5 ± 9.00*	10.4 ± 8.44**	2.6 ± 11.43**	0.05	0.01	0.59
Unfertilized oocytes per flush (n)	3.0 ± 1.90	9.0 ± 1.80	5.7 ± 2.35	0.06	0.05	0.23
Unfertilized oocytes per flush (%)	52.8 ± 11.95	84.9 ± 11.27	77.4 ± 15.12	0.18	0.09	0.70
Cows with more than 50% of fertilized structures[4] (%)	50.0 ± 16.40	11.1 ± 15.46	40.0 ± 20.75	0.25	0.27	0.28

*,**Values within the same row differ TRT (P 0.05). [1]NonSx = two AI with non-sorted semen (20.0 x 10^6 sperms/dose) 12 and 24 h after administering the LH (control group); Sx12&24 = two AI with sex-sorted semen (2.0 x 10^6 sperms/dose), 12 and 24 h after administering the LH; Sx24&36 = two AI with sex-sorted semen (2.0 x 10^6 sperms/dose) performed 24 and 36 h after administering the LH. [2]TRT = effects of treatment; Semen type = orthogonal comparison for the effects of semen type (NonSx *vs*. Sx12&24 + Sx24&36); AI Time = orthogonal comparison for the AI time with sexed semen. [3]Only the cows that responded to the superovulation protocol. [4]Proportion of the cows that showed more than 50% of fertilized structures.

Discussion

The use of sex-sorted semen in embryo production by SOV is an important tool for genetic improvement in dairy farms, consequently increasing milk production. This research evaluated the use of sexed semen in the *in vivo* embryo production during modifications at the time of artificial insemination. Our hypothesis that a delay at TAI using sex-sorted semen on the donors of embryo would increase the fertilization rate was rejected since the delay at the artificial insemination had no effect on the fertilization rate. The superovulatory response observed in this study showed that 18.51% (5/27) of the cows did not respond to the protocol that was used, which can be attributed to the variability of the female stimulatory treatments (Bo *et al*., 2006). The mean number of structures recovered per flush was similar to those reported in heifers (Sartori *et al*., 2004; Peippo *et al*., 2009) and Holstein cows (Peippo *et al*., 2009).

Although the sperm sex-sorting process has significantly evolved, when it is used in superovulated cows the fertilization rate is lower than when using non-sorted semen (Sartori *et al*., 2004; Larson *et al*., 2007, 2010; Soares *et al*., 2011; Kaimio *et al*., 2013). This result can be explained by the low sperm concentration and damage caused by the sorting process followed by freezing (dilution, laser exposure, process speed and centrifugation) (Schenk *et al*., 1999). In this study, the percentage of fertilized embryos per flush tended to be smaller when the sex-sorted semen was used rather than the non-sorted semen. Similar results have been reported previously (Kaimio *et al*., 2013). These results were observed even when greater sperm concentrations (10 x 10^6 and 20 x 10^6) were used in heat detection AI (Sartori *et al*., 2004), when timed AI (TAI) was used in heifers that were superovulated without an ovulation inductor (Schenk *et al*., 2006), when AI was conducted 6 h after the pre-established time (Soares *et al*., 2011), and when four inseminations were done with estrus observation (Larson *et al*., 2010).

Although no statistical differences were observed in the proportion of fertilized embryos when sex-sorted semen was used at different AI times, the number of transferable embryo per flush generally increased when the TAI was performed 24 and 36 h after LH administration when compared to 12 and 24 h. In a similar study (Soares *et al*., 2011), it was realized

that the use of TAI with sex-sorted semen in superovulated *Bos taurus* and *Bos indicus* cows resulted in good fertilization rates. In this case, the greatest numbers of freezable embryos were obtained when the inseminations were performed 18 and 30 h after LH administration, which corresponds to the findings of this study. Moreover, these results support the hypothesis that TAI with sex-sorted semen in superovulated cows should be performed during the period of greater ovulation occurrence to increase the fertilization rate.

Dalton *et al*. (2000) reported lower fertilization rates in superovulated cows when the AI was performed 0 or 12 h after the onset of estrus than when inseminated 24 h after (29.0, 60.0, and 81.0%, respectively). One possible explanation for failure in fertilization in superovulated cows seems to be the absence of viable sperm in the fertilization site (Schenk *et al*., 2006). After the AI period, possibly many sperm were removed from the female genital tract through retrograde loss and the loss of functional sperm reservoirs over the time span required for ovulation to occur (Dalton *et al*., 2000). The same effect can also be observed in single ovulation cows because higher pregnancy/AI is obtained when the interval between AI and ovulation is less than 16 hours when compared to intervals of 16-32 h and 32-48 h (50.8, 28.7, and 14.3%, respectively; Hockey *et al*., 2010).

Therefore, a lower number of transferable embryos were observed when the TAI of sex-sorted semen was performed 12 and 24 h after LH administration. This result can be attributed to two causes, 1) the reduction and/or absence of suitable sperm on the fertilization site at the time of ovulation and 2) the damage that sperm cells undergo during the sexing process, which determines the loss of some sperm functions, including motility, progressive motility and the number of cells with intact plasma and acrosomal membranes (Carvalho *et al*., 2010).

The unavailability of sex-sorted semen from another Brown-Swiss bull used in this study led us to focus on one individual bull. As individual variations occur between bulls in the same breed (Cochran *et al*., 2013), it is also known that the tolerance of bovine sperm to the sexing process by flow cytometry is variable (Alomar *et al*., 2008; DeJarnette *et al*., 2008; Garner, 2009) and requires a selection of individuals that have better AI results for non-sorted semen with low sperm concentrations.

In the present study, an increase in the number of degenerated embryos occurred in the NonSx group. This finding most likely resulted from the greater number of embryos produced in this group. However, the main hypothesis that explains these results involves heat stress. Unfortunately, the donors were exposed to adverse weather conditions (32.3°C temperature and 100% humidity) during the study period. Heat stress on dairy cattle can increase the number of degenerate embryos (Putney *et al*., 1989) and decrease the number of fertilized and freezable structures (Vieira *et al*., 2014). Lower fertilization rates occur because the heat stress effect compromises the oocyte quality and alters embryonic development (Edwards *et al*., 2009).

Although the amount of degenerated embryos potentially compromised the clarity of the data that were obtained in this study, the results suggest a path so that we can achieve better results in the superovulated dairy cows when using the sex-sorted semen.

In conclusion, these results demonstrate that the use of sex-sorted semen in superovulated dairy cows that were raised in tropical climate results in greater numbers of unfertilized oocytes than non-sorted semen. However, when only sorted semen is used, AI should be performed 24 and 36 h after administering the LH to obtain a greater number of transferable embryos.

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