

Enrichment of bovine semen with x-bearing spermatozoa using Percoll™ and Optiprep® discontinuous gradients

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Abstract: In cattle, sex selection has a significant economic impact when it improves herd capacity of milk or meat production. Density gradient centrifugation might be an approach to sexing spermatozoa because of the additional DNA content and volume of X-bearing sperm head. In the present work, the accuracy of sperm sexing by Percoll™ and OptiPrep® gradients centrifugation was compared. Bovine semen from three breeds (Holstein, Gir, and Red Angus) was used. The sperm viability and sexing accuracy after sperm selection by centrifugation of low and high-density discontinuous gradient composed by three layers of Percoll™ or OptiPrep® were assessed by *in vitro* embryo production (IVP) and pregnancy rate following artificial insemination (AI). After IVP, cleavage rate was higher ($P < 0.05$) for unsexed (81.3%) than for sexed (75.5%) spermatozoa. A significantly lower cleavage rate was observed for sexed (76.1%) spermatozoa when compared to sexed Red Angus (81.2%) spermatozoa. Additionally, significantly lower blastocyst rate was observed using Holstein sexed sperm by OptiPrep® gradient (23.3%) when compared with Gir (41.9%) and Red Angus (48.7%) sexed sperm by Percoll™ gradient. Nevertheless, in all breeds analyzed, a significant sex ratio deviation to females was observed. Analyzing the pregnancy rate after AI with sexed sperm by Percoll™ and OptiPrep®, semen from Gir bulls presented lower ($P < 0.05$) pregnancy/AI (61.3%) when compared to Red Angus (75.9%) and Holstein (77.4%) and bulls. From the results, it was concluded that the increase in female percentage after insemination and/or IVP with bovine sexed sperm by centrifugation of high density gradient composed by three layers of Percoll™ and OptiPrep® may allow this spermatozoa sexing technique to be used in large scale which would support genetic enhancement for milk and meat production as well as for progeny tests in cattle.

Keywords: Artificial Insemination, Bovine Sexed Semen, *In Vitro* Production

1. Introduction

In cattle, sex selection has a significant economic impact. It improves the herd capacity of milk and meat production [1; 2; 3].

Since bovine X-bearing sperm cells carry 3.8% more DNA than the Y-bearing spermatozoa, these two sperm types can be separated in two sperm subpopulations by flow cytometry [4;5] according to their differences in specific sperm head volume [6,7] and differences in the intensity of specific DNA-binding fluorochrome [4]. Alternatively, density gradient

centrifugation can be another approach to sperm sexing [8; 9; 10; 11; 12; 13] given that the extra DNA content and head volume of X-bearing spermatozoa is also related to a higher density of these cells, approximately 0.006% [14].

In bovines, reference [15] used two centrifugations protocols for separating X and Y sperm populations in discontinuous low density gradient of Percoll™. The gradients contained 10 layers of Percoll™ solutions (0.6 mL each layer) with density ranging from 1.034 to 1.068 g/mL at 6 °C. This method increased 75% of X-bearing and 65% of

Y-bearing sperm cells in bottom and upper fractions, respectively, which was confirmed by *in situ* hybridization. Reference [8] employing this same methodology for sperm selection, utilized the sperm cells from these two fractions for *in vitro* embryo production (IVP). The genetic sex of embryos was assessed by PCR (Polymerase Chain Reaction) and, according to the results, the use of sperm cells from upper and lower layers produced 75% and 92% of male and female embryos, respectively. A similar experiment was performed by reference [9] which observed, after *in situ* hybridization, 70% X-bearing spermatozoa in bottom fraction and 65% of Y-bearing spermatozoa in upper fraction.

In humans, reference [16] using low density gradient centrifugation at 6 °C, composed by 7 layers of Percoll™ solutions (1.050 to 1.123 g/mL) and one layer of Nycoprep™ (1.115 g/mL), detected by *in situ* hybridization 61% of X-bearing spermatozoa in the lower fraction. Recently, reference [13] were able to separate 60% of X-bearing human spermatozoa using low density gradient centrifugation at room temperature with a gradient composed by 8 layers of 38% to 84% of albumin.

However, despite those interesting results reported above, this sexing methodology has never been used as a common tool in animal reproductive procedures, mainly due to the limited success and the low repeatability of such bio technique. Hence, the aim of the present study was to evaluate if bovine sperm centrifugation on high and low discontinuous density gradients composed by three layers of Percoll™ or OptiPrep® are able to increase the X-bearing sperm population in the bottom layer of the gradient.

2. Experimental Development

All reagents used in this study were obtained from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated.

2.1. Semen Collection and Quality Control

For all trials, semen was collected from 10 bulls of three breeds (ranging from 4 to 8 years old, 2 Holstein Friesian, 4 Gir and 4 Red Angus) in a routine collection schedule using an artificial vagina. After collection, sperm concentration was determined by a hemocytometer in a 1:200 dilution.

The percentages of progressive motile sperm and vigor were determined subjectively under a bright-field microscope at 400X magnification. Only ejaculates with a concentration higher than 900×10^6 sperm/mL, a progressive motility of at least 60% and vigor between 4 to 5 were processed. Thereafter, 3mL samples from 75 ejaculates were divided in half to produce sexed and non-sexed sperm.

Procedures for sexing, processing and freezing Percoll™ discontinuous density gradient

Percoll™ (Pharmacia Fine Chemicals, Uppsala, Sweden) stock solution (PSS; density 1.123 g/mL) was prepared adding 9 parts (v/v) of Percoll™ (density 1.130 g/mL) to 1 (v/v) of 10 X concentrated (DMEM 10 X) containing 0.01 g/L gentamicin sulfate; 6mM HEPES; pH 7.4; 280-320 mOsm/kg of H₂O. To prepare the discontinuous density gradient, PSS was diluted with DMEM 1 X (containing 0.01 g/L of gentamicin sulfate; 6

mM of HEPES; 0.3% bovine serum albumin V fraction, BSA (Calbiochem, Darmstadt, Germany; pH 7.4) to produce different densities.

Gradient 1 (G1) was prepared to be used as a control group. It was composed by 10 layers of Percoll™ solutions (1 mL each layer), with the following densities: 1.034 g/mL, 1.038 g/mL, 1.042 g/mL, 1.045 g/mL, 1.049 g/mL, 1.053 g/mL, 1.057 g/mL, 1.059 g/mL, 1.064 g/mL, 1.068 g/mL.

Gradient 2 (G2) was composed by 3 layers of 3mL each of Percoll™ solutions of densities ranging from 1.110 g/mL to 1.123 g/mL.

The Percoll™ discontinuous gradient was prepared by layering each solution of Percoll™ from the densest to the lowest in 15 mL centrifuge tubes (Corning, 430053, New York, USA), with the help of an adjustable pipette with a reservoir for automated volume dispenser (LabSystems, 4540, Helsinki, Finland).

2.2. OptiPrep® Discontinuous Density Gradient

The OptiPrep® (Axis-Shield, 1114542, New York, USA) was diluted in DMEM 1X (D1152, Sigma-Aldrich, St Louis, MO, USA) medium in different proportions to produce different densities solutions.

Gradient 3 (G3) was composed by 3 layers of OptiPrep® solution with 3 mL each, of densities ranging from 1.123 g/mL to 1.163 g/mL.

Gradient 4 (G4) was composed by 10 layers of 1 mL each of Optiprep™ solutions with densities: 1.034 g/mL, 1.038 g/mL, 1.042 g/mL, 1.045 g/mL, 1.049 g/mL, 1.053 g/mL, 1.057 g/mL, 1.059 g/mL, 1.064 g/mL, 1.068 g/mL.

The discontinuous gradient of OptiPrep® was prepared by layering each OptiPrep® solution, from the densest to the lowest, in 15 mL centrifuge tubes (Corning, 430053, New York, USA), with the help of an adjustable pipette with a reservoir for automated volume dispenser (LabSystems, 4540, Helsinki, Finland).

2.3. Spermatozoa Centrifugation in Discontinuous Density Gradients

Aliquots of 1.0 mL of semen containing 90×10^6 spermatozoa were layered gently on each discontinuous density gradient that was then centrifuged in a bench-top centrifuge at $500 \times g$ for 15 minutes, at 22°C. Next, the supernatant was discarded using a Pasteur pipette. Pellets were resuspended with 200 µL of Tris-egg yolk extender containing 1.4 M glycerol. Sperm concentration was adjusted to 12×10^6 spermatozoa/straw.

2.4. Further Processing and Freezing

Sexed or non-sexed sperm of each ejaculate was cooled at 5°C for 1-2 hours and packaged in 0.25 mL French straws (IMV, Aigle, France), each containing 12×10^6 spermatozoa. After packaging, straws were kept in freezing racks and frozen in an automatic freezer (IMV, Aigle, France) and stored in liquid nitrogen at -196 °C. Three days after freezing, one straw of each batch was thawed at 35°C for 20 sec and quality parameters

were established. The minimum values obtained immediately after thawing (0 hours) were 30% progressive motility and vigor 4; and after the resistance test (3 hours) were 20% spermatozooids with progressive motility and vigor 3.

2.5. Experiment 1 - In vitro Embryo Production Using Spermatozoa Sexed by Density Gradient Centrifugation

Ovaries collected at slaughterhouse were transported to laboratory in saline solution at 33°C. Immature cumulus oocyte complexes (COCs) were aspirated from follicles with a diameter of 3 and 6 mm. The COCs were washed in TCM-199, supplemented with 0.2 mM of sodium pyruvate, 20 mM of HEPES, 5 mM of sodium bicarbonate, and 16.67 µg/mL of amikacin. The oocytes were matured in TCM-199 with 0.2 mM of sodium pyruvate, 25 mM Na₂HCO₃, 16.67 µg/mL amikacin, 1 µg/mL 17-β estradiol, 0.5 µg/mL follicle stimulating hormone (FSH; Folltropin™, Bioniche Animal Health, Belleville, USA), 5.0 µg luteinizing hormone (LH; Lutropin-V, Vetefarm, London, UK), and 10% (v/v) fetal calf serum (FCS; Gibco-BRL, Grand Island, NY, USA). The COCs were matured in 100 µL droplets (20 oocytes per droplet) under mineral oil at 38.5 °C in 5% CO₂ humid atmosphere for 22 hours.

For IVP of bovine embryos, matured oocytes were inseminated with frozen-thawed non-sexed or sexed semen. In each group, the straws were thawed at 35 °C for 30 sec and layered on top of a gradient composed by 1 mL fraction each of 45% and 90% isotonic Percoll™. After 30 min centrifugation at 900 x g the supernatant was discarded and the pellet adjusted with fertilization medium to obtain a final concentration of 100 x 10³ spermatozoa in each fertilization droplet. The oocytes and sperm were incubated for 20 h in 5% CO₂, in humidified air at 38.5 °C, in TALP-IVF medium. Presumptive zygotes were denuded and co-cultured with synthetic oviduct fluid (SOF) medium. They were then transferred to four-well dishes, and the embryo culture was done at 38.5°C in a humid atmosphere containing 5% CO₂, 5% O₂ and 90% N₂ [17]. The cleavage was assessed 48 h after IVF and the blastocyst at 7 to 8 days of culture. Fetal calf serum (FCS) was not added during *in vitro* culture, as previously recommended [18]. Embryos in blastocyst stage were frozen individually into microtubes containing 10µL of double distilled water and stored at -20 °C for further determination of the genetic sex by PCR.

2.6. Determination of Embryo Genetic Sex by PCR

Before amplification reactions, a Proteinase K (Gibco-BRL, CA, USA) digestion of each embryo was performed. Each embryo received 5µg of Proteinase K and was kept at 37 °C for 1 hour; the Proteinase K was then denatured at 98°C for 10 min.

Each sample was amplified by two distinct and independent reactions, named A and B. Reaction A consisted in the simultaneous amplification of two Y-chromosome specific segments using primer pairs 1 (forward 5'-CCTCCCCTTGTTCAAACGCCCGGAATCATT-3' and

reverse 5'-TGCTTGACTGCAGGGACCGAGAGGTTTGGG-3') and 2 (forward 5'-ATCAGTGCAGGGACCGAGATG-3' and reverse 5'-AAGCAGCCGATAAACACTCCTT-3'). Primer pairs 1 and 2 amplified a 210 bp [19] and a 250 bp [20] product in bovine males, respectively; reaction B, by its turn, amplified a 280 bp product from a repetitive sequence of the bovine genome used as internal control (primer pair 3: 5'-AGGTCGCGAGATTGGTCGCTAGGTCATGCA- 3' and 5'-AAGACCTCGAGAGACCCCTCTTCAACACGT-3';) [21]. Thus, female samples produced only one band of 280 bp resulted from the amplification reaction B while male samples produced 3 bands of 280 bp and 250 bp (reaction A) and of 210 bp (reaction B).

Amplification reactions were performed in 10mM Tris-HCl buffer with 1mM of EDTA, pH 8; 2mM MgCl₂, 1U of Taq DNA Polymerase (AmpliTaq Gold, Applied Biosystem, Forrest City, CA, USA) and 10 pmol of each primer. Reactions A were submitted to 40 cycles of 94°C for 60 sec, 58°C for 30 sec, and 72°C for 60 sec, followed by 7 min at 72°C. Reactions B were submitted to 38 cycles of 94°C for 60 sec, 58°C for 60 sec, and 72°C for 60 sec, followed by 7 min at 72°C.

Amplified products were visualized in a 5% agarose gel and analyzed with Stratagene software (Eaglesight® Software).

2.7. Experiment 2 - Artificial Insemination Using Spermatozoa Sexed by Percoll™ or OptiPrep® Discontinuous Density Gradient Centrifugation

A total of 390 extensively pasture raised heifers (*Bos indicus* X *Bos Taurus*), with age ranging from 24 and 36 months, were inseminated for the present experiment. The heifers presented body condition score higher than 4.5 in a scale from 1 (emaciated) to 9 (extremely fat). Estrus detection and AI were performed by two experienced technicians. Heifers were inseminated following natural estrus [22] with semen sexed by gradients G1 or G2, or with non-sexed semen from the same ejaculate.

2.8. Fetal Sexing by Ultrasound

Transrectal ultrasonography was performed 60 to 70 days after AI to evaluate pregnancy rates (pregnancy per AI) and to identify fetal gender using a Pie Medical Scanner (200 apparatus with linear transducer of 5 MHz), according to the criteria described by reference [23].

2.9. Statistical Analysis

The effect of sexing or breed on cleavage and blastocyst rates (Experiment 1) was analyzed with multiple comparison Analyses of Variance (ANOVA) followed by the Tukey test of Multiple Comparisons. Data were expressed as percentages on mean ± SD. Pregnancy rate and fetal sex (Experiment 2) were analyzed by Chi-square (X²).

3. Results

Regarding the proportion of sperm recovered from the bottom of density gradients after centrifugation in Percoll™ and Optiprep® gradients, no difference was observed among the groups ($P > 0.05$; a total of 13% in Percoll™ and 10% in Optiprep® gradients; $n = 75$ ejaculates from 10 bulls). The recovered sperm was used to produce 439, 406, and 368 doses of sexed sperm of Gir, Holstein, and Red Angus breeds, respectively (total of 1213 sexed doses).

Regarding semen quality after thawing, it was observed that the sexing procedure reduced ($P < 0.05$) sperm viability. In non-sexed semen, 100% of 75 ejaculates presented a minimum sperm motility of 50% and vigor 4. In sexed semen, 66.7% (50 of 75) of the batches presented a minimum sperm motility of 50% and vigor 4. After sperm thermal resistance test (TTR), in non-sexed semen, 84% (63/75) of the batches presented a minimum sperm motility of 20% and vigor 3. In sexed semen, after TTR, 20.6% of the batches presented a minimum sperm motility of 20% and vigor.

3.1. Experiment 1 - In Vitro Embryo Production Using Sexed Spermatozoa by Density Gradient Centrifugation

Cleavage and blastocyst rates were calculated considering the total number of oocytes used for each gradient used, per breed.

No difference on cleavage rate was observed between high and low density gradients of Percoll™ and Optiprep®. For all breeds, sexed spermatozoa had an effect ($P < 0.01$) on cleavage rate of IVP. Using non-sexed sperm, cleavage rate (81.3%) was higher ($P < 0.05$) than using sexed sperm (75.5%) (Table 1).

Table 1. Means of cleavage and blastocyst rates of in vitro produced bovine embryos using 75 batches of spermatozoa either non-sexed or sexed by Percoll™ (G1 and G2)^a or OptiPrep® (G3 and G4)^b discontinuous density gradient centrifugation from semen of Gir, Holstein, and Red Angus bulls.

Treatment	Total number of oocytes	Cleavage rate* (% ± SD)	Blastocyst rate** (% ± SD)
Unsexed	5115	81.0 ± 7.9	38.1 ± 11.8
Sexed	5963	75.6 ± 13.1	33.4 ± 13.7

* $P < 0.0001$ (Chi-square)

** $P = 0.0023$ (Chi-square)

^aG1: densities from 1.110 g/mL to 1.123 g/mL; G2: densities from 1,034 to 1,045 g/mL

^bG3: densities from 1.123 g/mL to 1.163 g/mL; G4: densities from 1,034 g/mL to 1,045 g/mL

Table 2. Breed effect on cleavage rate means of in vitro produced bovine embryos using 75 baths of spermatozoa sexed by Percoll™ (G1 and G2)^a or OptiPrep® (G3 and G4)^b discontinuous density gradient centrifugation from semen of Gir, Holstein, and Red Angus bulls.

Breed	Total number of oocytes	Cleavage rate (% ± SD)
Gir	3011	76.1 ± 13.4 ^c
Holstein	4776	77.7 ± 8.2 ^{cd}
Red Angus	3291	81.2 ± 11.7 ^d

^aG1: densities from 1.110 g/mL to 1.123 g/mL; G2: densities from 1,034 to 1,045 g/mL

^bG3: densities from 1.123 g/mL to 1.163 g/mL; G4: densities from 1,034 g/mL to 1,045 g/mL

^{c-d} Means without common superscript letters differ $P < 0.05$ (Chi-square).

Breed also had an effect on cleavage rate ($P < 0.05$). The cleavage rate obtained from sexed semen of Red Angus (81.2%) was significantly higher when compared to cleavage rate obtained from sexed semen of Gir (76.1%) (Table 2).

Gradient centrifugation had a significant effect ($P > 0.01$) on blastocyst rate, as shown by comparing means of blastocysts obtained from sexed (35.0%) and non-sexed (38.1%) sperm (Table 1).

Table 3. Breed effect on blastocyst rate means of in vitro produced bovine embryos using 75 baths of spermatozoa sexed by Percoll™ (G1 and G2) or OptiPrep® (G3 and G4) discontinuous density gradient centrifugation from semen of Gir, Holstein, and Red Angus.

Breed	Gradient ^a	Blastocyst rate (% ± SD)
Gir	G1	42.0 ± 13.3 ^b
	G2	37.4 ± 11.5 ^b
	G3	41.9 ± 12.5 ^b
	G4	36.9 ± 12.8 ^b
Holstein	G1	30.1 ± 11.8 ^b
	G2	29.9 ± 9.7 ^b
	G3	23.2 ± 9.8 ^b
	G4	36.9 ± 12.2 ^b
Red Angus	G1	36.6 ± 14.5 ^{bc}
	G2	37.4 ± 11.4 ^{bc}
	G3	48.6 ± 12.4 ^b
	G4	30.0 ± 7.5 ^c

^aG1: densities from 1.110 g/mL to 1.123 g/mL; G2: densities from 1,034 to 1,045 g/mL; G3: densities from 1.123 g/mL to 1.163 g/mL; G4: densities from 1,034 g/mL to 1,045 g/mL.

^{b-c} Means without common superscript letters differ, $P < 0.05$ (Test Tukey)

There was an association ($P < 0.001$) between breed and sexing for blastocyst rate. The Tukey test for sexing and breed allowed pair-wise comparison of all means, considering either breed or discontinuous density gradient (Tables 3 and 4).

Table 4. Gradient effect on blastocyst rate means of in vitro produced bovine embryos using 75 baths of spermatozoa sexed by Percoll™ (G1 and G2) or OptiPrep® (G3 and G4) discontinuous density gradient centrifugation from semen of Gir, Holstein, and Red Angus bulls.

Gradient ^a	Breed	Blastocyst rate (% ± SD)
G1	Gir	42.0 ± 13.3 ^b
	Holstein	30.1 ± 11.8 ^b
	Red Angus	36.6 ± 14.5 ^b
G2	Gir	37.4 ± 11.5 ^b
	Holstein	29.9 ± 9.7 ^b
	Red Angus	37.4 ± 11.4 ^b
G3	Gir	41.9 ± 12.5 ^b
	Holstein	23.2 ± 9.8 ^c
	Red Angus	48.6 ± 12.4 ^b
G4	Gir	36.9 ± 12.8 ^b
	Holstein	36.9 ± 12.2 ^b
	Red Angus	30.0 ± 7.5 ^b

^aG1: densities from 1.110 g/mL to 1.123 g/mL; G2: densities from 1,034 to 1,045 g/mL; G3: densities from 1.123 g/mL to 1.163 g/mL; G4: densities from 1,034 g/mL to 1,045 g/mL

^{b-c} Means without common superscript letters differ, $P < 0.05$ (Test Tukey)

When breed was taken into consideration was observed that, in Red Angus, the blastocyst rate was significantly higher when the sexed semen obtained with gradient 1. No significant difference was observed with the other breeds (Table 3). When gradient was considered, was observed that sexed semen obtained from gradient 1 significantly decreased ($P < 0.05$) blastocyst rate in Holstein (Table 4). However, for the other gradients, this effect was not significant.

Regarding the sex ratio among embryos produced in vitro, the variance analysis detected an effect of sperm sexing on sex ratio. An increased female proportion was observed after IVP with sexed semen (72.3%) compared to the female proportion after IVP with non-sexed semen (48.2%) (Table 5). No difference was observed between high and low density gradient centrifugation on female proportion.

Experiment 2 - Artificial insemination using spermatozoa sexed by density gradient centrifugation

A total of 162 crossbred heifers were inseminated with frozen-thawed non-sexed semen of Gir, Red Angus and Holstein breeds; whereas 228 crossbred heifers were inseminated with frozen-thawed sexed semen of Gir, Red Angus, and Holstein breeds, from either G2 or G4. Tables 6 and 7 present the pregnancy rates and sex ratios obtained 60 days following AI with non-sexed semen and with sexed semen from G2 and G4.

Table 5. Means of female in vitro produced bovine embryos using 75 thawed batches of spermatozoa either non-sexed or sexed by Percoll™ (G1 and G2)^a or OptiPrep® (G3 and G4)^b discontinuous density gradient centrifugation from semen of Gir, Holstein, and Red Angus bulls.

Treatment	Total number of embryos sexed by PCR	Female embryos*(% ± SD)
Non-sexed	3338	48.2 ± 4.4
Sexed	3605	72.3 ± 10.4

* $P < 0.0001$

^aG1: densities from 1.110 g/mL to 1.123 g/mL; G2: densities from 1,034 to 1,045 g/mL

^bG3: densities from 1.123 g/mL to 1.163 g/mL; G4: densities from 1,034 g/mL to 1,045 g/mL

Table 6. Pregnancy rates in crossbred heifers and female fetus 60 days after AI using semen either non-sexed or sexed by Percoll™ discontinuous density gradient (G1 and G2) from semen of Gir, Holstein, and Red Angus bulls.

Breed	Treatment	Total number of heifers inseminated	Pregnancy rate (%)	Female fetus (%)
Gir	Non-sexed	31	67.7c	10 (47.6)d
	Sexed	32	61.3c	13 (68.4)e
Holstein	Non-sexed	30	86.7b	13 (50.0)d
	Sexed	31	77.4b	17 (70.8)e
Red	Non-sexed	30	76.7b	12 (52.2)d
Angus	Sexed	29	75.9b	16 (68.1)e

^aG1: densities from 1.110 g/mL to 1.123 g/mL; G2: densities from 1,034 to 1,045 g/mL

^{b-c} Means without common superscript letters differ, $P < 0.05$ (Chi-square)

^{d-e} Means without common superscript letters differ, $P < 0.05$ (Chi-square)

Table 7. Pregnancy rates in crossbred heifers and female fetus 60 days after AI using semen either non-sexed or sexed by OptiPrep® discontinuous density gradient (G3 and G4)^a from semen of Gir, Holstein, and Red Angus bulls.

Breed	Treatment	Total number of inseminated heifers	Pregnancy rate (%)	Female fetus (%)
Gir	Non-sexed	20	65.0c	7 (53.9)d
	Sexed	37	59.5c	18 (81.8)e
Holstein	Non-sexed	28	75.9b	23 (57.5)d
	Sexed	54	85.7b	17 (70.8)e
Red	Non-sexed	23	73.9b	9 (52.6)d
Angus	Sexed	45	80.0b	26 (72.2)e

^aG3: densities from 1.123 g/mL to 1.163 g/mL; G4: densities from 1,034 g/mL to 1,045 g/mL

^{b-c} Means without common superscript letters differ, $P < 0.05$ (chi-square)

^{d-e} Means without common superscript letters differ, $P < 0.05$ (chi-square)

The sexing procedure had no significant effect on pregnancy rate when compared to non-sexed semen of the same breed 60 days following AI. However, there was a significant decrease ($P < 0.05$) in pregnancy rate obtained with sexed and non-sexed semen of Gir breed if compared to sexed and non-sexed pregnancy rate obtained with Holstein and Red Angus breeds. Notably, AI with sexed semen of Red Angus and Holstein breeds seemed to present higher ($P < 0.05$) pregnancy rates when compared to Gir breed. A significant sex ratio deviation ($P < 0.05$) toward to female gender was observed when AI was performed with sexed semen obtained with all density gradients in all breeds (Table 7). The sex ratio deviation ($P < 0.05$) of 70.7% in favor of female gender was confirmed at parturition. No occurrences of dystocia, or neonatal mortality were reported.

4. Discussion

In this work, a method for sexing bovine sperm with high and low density discontinuous gradient made with isotonic solutions from modified colloidal silica (Percoll™) or iodinated compounds (OptiPrep®) was used. The composition of these gradients allowed a lower accuracy (70%) when separating X-bearing bovine spermatozoa than that observed by reference [8] (90%), reference [9] (70%), and reference [15] (75%). After thawing, 66.7% of the straws had at least 50% of sperm with motility that produced on average 33.4% blastocysts with IVP of bovine embryos and 73% pregnancy rates after conventional artificial insemination.

Considering the samples of 75 ejaculates in one of the four gradients, on average of 16 doses of semen (12×10^6 sperm per straw) enriched with X-bearing sperm in each 2 mL of the ejaculate were produced every 30 minutes, using approximately 30% of each ejaculate, distributed in 36 tubes.

The discontinuous Percoll™ and OptiPrep® density gradients were employed for the selective isolation of bovine X-bearing spermatozoa. Retrieval of spermatozoa with Y chromosome in the Percoll™ and OptiPrep® discontinuous gradients was on average 5% (data not shown), as the Y chromosomes were spread out in gradients similarly to what is observed with human species [24; 25] and with bovines [8]. This low recovery of Y-bearing spermatozoa is not a relevant issue

because female selection is more relevant for commercial milk and meat production from pure breed as well as for progeny tests and genetic enhancement for milk and meat production [1; 2].

The use of density gradient centrifugation to sort bovine sperm in IVP systems had been investigated in previous studies [8; 9] and provided similar results to those here presented.

The fact that was used a large number of samples (6943 embryos, 13,886 PCRs) in this study, provided great power to our statistical analysis and allowed us to observe the great deviation in sex ratio that sexing may cause. To our knowledge, the only description of embryos sexed by density gradient typed 20 embryos [8]. Interestingly, embryos sexed by reference [8] had their sex confirmed by PCR and produced 90% females, which is higher than the percentage found in this study (table 6). Besides the fact that these 20 embryos were produced with a single enriched straw of sperm and in a single procedure of IVP embryos, the greater number of embryos may have led to detect less deviation.

Cleavage and blastocyst rates in this study were higher than those described by reference [8], who found 53.4% cleavage and 20.2% blastocysts in 515 oocytes using the Percoll™ gradient centrifugation. Another noteworthy fact of their study is that the IVP did not produce over 50% male embryos, as observed in the control groups and expected to be obtained in this type of procedure. Using bovine fetal serum instead of BSA and eliminating glucose during embryo development may explain these observations. Reference [26] showed that when glucose is present, more than 75% of blastocysts produced in SOF medium were male. In SOF medium containing SFB not only was the development rate accelerated but also survival rates of male embryos increased [18].

Glucose in high concentrations not only impairs development of bovine embryos but also causes a selective block of female embryos during transition from morula to blastocyst, which leads to a higher rate of males among the expanded blastocysts [27]. This phenomenon is likely to be explained by the presence in female embryos of the two active X chromosomes, which have low tolerance for imbalances in the carbohydrate metabolism [27].

The results obtained after insemination of heifers with sperm sorted by discontinuous density gradient centrifugation suggest that the process did not affect *in vitro* fertility process nor had an effect after artificial insemination. The pregnancy rate obtained with OptiPrep® was 75.06%.

No reports of AI done in bovines using sperm centrifuged in either Percoll™ or OptiPrep® density gradient were found. The results show in this study showed that it is possible to produce pregnancies using conventional artificial insemination of semen sexed by density gradient centrifugation. In addition, it was possible to observe that this sexing method was compatible with freezing, it minimized spermatozoa loss during the process, and did not reduce pregnancy rates.

It is also important to evaluate sexing methodologies not only on their ability to precisely determine embryo sex. It is also important to evaluate the ability of sexed spermatozoa to

produce pregnancies. These issues are most relevant when implementing sexing methodologies on a large scale, so that neither reproduction rates nor the genetic enhancement obtained with production of milk and meat systems are affected.

Improvements in density gradient centrifugation, especially in its precision, may allow this system of spermatozoa sexing to be used on a large scale for milk and meat production as well as for progeny tests in bovines.

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