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The effect of sperm selection by Percoll or swim-up on the sex ratio of *in vitro* produced bovine embryos

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Abstract

The goal of this investigation was to determine the percentage of male and female bovine embryos produced after oocyte fertilization with Percoll density gradient centrifugation or with self-migration (swim-up) selected semen. In Experiment 1, sperm selection was performed by 90-45% discontinuous Percoll density gradient centrifugation (T1) and swim-up (T2). In Experiment 2, in addition to the discontinuous gradient, a 67.5% continuous gradient, and 5 or 10 min centrifugation were used. Four treatment groups were defined (TI = continuous, 5 min, TII = discontinuous, 5 min, TIII = continuous, 10 min and TIV = discontinuous, 10 min). Embryos were sexed using PCR. In Experiment 1, Percoll density gradient centrifugation (n = 185) resulted in 48.6% (n = 90) male and 51.4% (n = 95) female embryos, and swim-up (n = 142) in 58.4% (n = 83) male and 41.6% (n = 59) female embryos. In Experiment 2, the percentages of male and female embryos obtained in TI (n = 93), TII (n = 70), TIII (n = 82) and TIV (n = 82) were 49.5% (n = 46) and 50.5% (n = 47), 57.1% (n = 40) and 42.9% (n = 30), 36.6% (n = 30) and 63.4% (n = 52) and 48.8% (n = 40) and 51.2% (n = 42), respectively. Swim-up (T2) and continuous Percoll gradient centrifugation for 10 min (TIII) showed a deviation toward males (P = 0.044) and females (P = 0.015), respectively, when compared to the 50% expected percentage of each gender. There was no difference in male and female percentages in the other treatment groups from Experiments 1 and 2, when compared to the 50% expected percentage of each gender neither between treatments.

Keywords: density gradient, DNA, IVF, self-migration; X- and Y-chromosomes.

Introduction

Advances in DNA technology have enhanced the perspectives of genetic selection in domestic species to increase productivity (Garcia, 2001). Predetermining the gender of offspring in the dairy and beef cattle industry allows the breeders to plan their production toward a specific gender. The most effective way to achieve this goal is separating X- from Y-bearing spermatozoa (Stap *et al*., 1998). According to Cran and Johnson (1996), the only way to effectively separate sperm cell populations is based on their DNA content (Stap *et al*., 1998). Flow cytometry has been widely used for this purpose (Johnson, 2000; Bodmer *et al*., 2005; Garner, 2006), however, this technique presents some specific problems, such as broad fluorescence distribution without a distinct X- and Y- peak (Stap *et al*., 1998).

In an attempt to develop a method for the separation of sperm cell populations based on their DNA content, Percoll density gradient centrifugation has been used on human and bovine sperm (Kaneko *et al*., 1983, 1984; Hossepian-de-Lima *et al*., 2000). Percoll is composed of colloidal silica particles (15-30 nm in diameter) coated with polyvinylpyrrolidone (Samardzija *et al*., 2006), which increases the specific gravity of the medium to 1.13 g/ml (Makler *et al*., 1998). It is also used to isolate bacteria (Leuschner *et al*., 1999), neutrophils (Woldehiwet *et al*., 2003), viruses (Hanabusa *et al*., 2000) and subcellular particles (Swales and Wright, 2000; Domart-Coulon *et al*., 2001; Sheoran *et al*., 2005).

Discontinuous 90-45% Percoll density gradient centrifugation is widely used to increase sperm motility (Parrish *et al*., 1995; Alvarenga and Leão, 2002; Suzuki *et al*., 2003) for *in vitro* fertilization (IVF) and artificial insemination (AI). Swim-up is another commonly used sperm selection method for IVF (Parrish *et al*., 1986, 1995; Palomo *et al*., 1999). It is a simple and cheap method (Rheingantz *et al*., 2002; Henkel and Schill, 2003) which selects highly motile spermatozoa that reach the medium surface after incubation.

Considering the simplicity and practicality of these sperm selection methods, the purpose of the present study was to determine if semen selected by swim-up, 90-45% discontinuous and 67.5% continuous Percoll density gradient centrifugation alter the gender percentage of *in vitro* produced bovine embryos. With the same purpose, a 5 and a 10 min centrifugation was tested on both Percoll gradients.

Materials and Methods

Experiment 1

Immature oocytes were recovered from bovine ovaries obtained at a local slaughterhouse and kept in sterile 0.9% saline solution at 30 to 35ºC. Cumulus-oocyte-complexes (COCs) were recovered by aspiration

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from 2 to 8 mm diameter follicles using an 18-ga needle attached to a vacuum pump (20 ml/min pressure). Search, selection (De Loos *et al*., 1989) and maintenance of COCs was performed in follicular fluid according to Lehmkuhl *et al*. (2000). Only COCs with homogeneous cytoplasm and completely covered by compact and unexpanded cumulus oophorus cells were selected and randomly distributed in the experimental treatment groups.

Immature oocytes were placed on 4-well Nunc® dishes. Each well contained 400 µl of TCM 199 maturation medium supplemented with 26.2 mM $NaHCO_3$, 25 mM Hepes, 0.2 mM sodium pyruvate, 0.01 UI/ml FSH, 5 µg/ml LH and 10% estrus cow serum (ECS) covered with mineral oil. Maturation was performed for 20 to 24 h in an incubator at 38ºC, 5% CO_2 atmosphere and 95% humidity. On each routine, oocytes (average of 30 oocytes/well) were equally distributed between experimental groups.

After maturation, oocytes were transferred to new sterile 4-well Nunc® dishes containing 400 µl of Talp-Fert fertilizing medium (supplemented with 50 µg/ml heparin), covered with mineral oil. Frozen semen from one *Bos taurus* bull with proven *in vitro* fertilization (IVF) fertility was thawed in a water bath at 37°C for 20 s.

A 90-45% discontinuous Percoll density gradient (Parrish *et al*., 1995) was prepared, adding 1 ml of each Percoll fraction in a conic 15 ml tube. This gradient was used for treatment group 1 (T1). For sperm selection, 500 µl of thawed semen was layered on the top of the gradient and centrifuged at 120 x g for 10 min. The supernatant was discarded, the pellet recovered, suspended in 4 ml of Talp-Sperm and centrifuged at 50 x g for 10 min to remove residual Percoll. Two-hundred microliters from the washed pellet was recovered.

In group 2 (T2), the self-migration (swim-up) technique described by Parrish *et al*. (1986) was used. Three semen fractions of 100 µl each were placed on the bottom of three conic tubes containing 1 ml of Talp-Sperm medium. Tubes were kept at 38ºC immersed in a water bath for 1 h. Eight-hundred microliters from the upper Talp-Sperm medium layer were transferred to 4 ml Talp-Sperm and centrifuged 10 min at 50 x g. Two-hundred microliters from the pellet was recovered.

Fertilization was performed using 1 to 2 x 10^6 spermatozoa/ml in the fertilization medium containing oocytes. The gametes were incubated for 18 to 22 h at the same conditions as maturation.

Presumptive zygotes were vortexed for 1.5 min and washed with TCM-Hepes. Then they were transferred to SOFaaci culture medium (supplemented with 5% ECS and 0.022 mg/ml sodium pyruvate) and covered with mineral oil. On day 2 (D2) of culture (day zero; D0 = IFV day), fertilization ratio was evaluated by identifying cleavage. Embryos were cultured for four days in the foil bag system according to Vajta *et al*. (1997) at an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. On the fifth day (D5) of culture, embryo morphology was evaluated and they were classified according to their developmental stage (International Embryo Transfer Society, 1998) and prepared for sexing.

Morulae were washed in BSA-free PBS with polyvinilalcohol (PVA). Embryos were individually transferred to 0.2 ml PCR tubes previously filled with 10 µl of ultrapure water, immersed in liquid nitrogen for 30 s and stored at -20ºC, pending DNA extraction. Therefore, embryos were treated with proteinase K (16 mg/ml) at 37°C for 1 h followed by 98ºC for 10 min. Embryo DNA was divided into two samples for two PCR rounds (reaction 1 and 2). Reaction 2 confirmed results of reaction 1.

To obtain DNA control samples, 5 ml of blood were collected from male and female cattle through jugular vein puncture. Blood was kept in 15 ml glass tubes with 100 µl of 5% EDTA and stored at -20ºC. For extraction, 10 µl of 20% SDS and 15 µl of proteinase K (1 mg/ml) were added to 100 µl of blood and the mixture was incubated at 55ºC for 2 h. Phenol was added (v:v) to the mixture and the tube was vortexed and then centrifuged for 5 min at 8000 x g. This procedure was repeated with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1). The supernatant was mixed with 0.5 volume of 7.5 M ammonium acetate and 2 volumes of 95% cold ethanol and centrifuged for 10 min at 8000 x g. The pellet was washed twice with 250 µl of 70% cold ethanol and suspended in 500 µl of ultrapure water.

Three specific primer pairs designed to amplify only bovine DNA were used. One primer pair, called BOV, amplifies a 280 bp region of an autosomic gene from bovine DNA, which sequences are 5' – AGG TCG CGA GAT TGG TCG CTA GGT CAT GCA – 3' and 5' – AAG ACC TCG AGA GAC CCT CTT CAA CAC GT – 3'. Y1 and Y2 primer pairs amplify a 210 bp and a 250 bp region, respectively, only from male bovine DNA. Y1 sequences are 5' – CCT CCC CTT GTT CAA ACG CCC GGA ATC ATT 3' and 5' – TGC TTG ACT GCA GGG ACC GAG AGG TTT GGG – 3 and Y2 sequences are 5' – ATC AGT GCA GGG ACC GAG ATG – 3' and 5' – AAG CAG CCG ATA AAC ACT CCT T – 3'. BOV and Y1 primers were used in the same PCR round (reaction 1), while Y2 primers were used in reaction 2. Reaction conditions were: 75 mM Tris-HCl pH 9, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 160 µM dNTPs, 0.17 µM of each primer and 1U Taq DNA polymerase. Reactions had a final volume of 30 µl. Positive (male and female DNA extracted from bovine leukocytes) and negative (water) controls were used in all PCR reactions. All DNA samples were denatured at 94ºC for 5 min in a PTC-100™ thermal cycler. Reaction 1 ran 40 cycles at 94ºC for 60 s, 58ºC for 30 s and 72ºC for 60 s, followed by an additional 7 min at 72ºC. Reaction 2 ran 38 cycles at 94ºC for 60 s,

58ºC for 60 s and 72ºC for 60 s, followed by an additional 7 min at 72^0C.

Amplification products were loaded on 2% agarose gels stained with ethidium bromide and visualized on a UV lightbox. Electrophoresis was run at 90V, 100W, 150 mA for 1.5 h. Female embryos were identified by the detection of a 280 bp band on reaction 1 and no band on the corresponding reaction 2. The presence of 2 bands (280 bp and 210 bp) on reaction 1 indicated a male embryo, and this was confirmed by the identification of a 250 bp band on the corresponding reaction 2.

Experiment 2

Oocyte recovery, IVM and sperm preparation were performed as described in Experiment 1. IVM and IVF, however, were performed in 200 µl drops of maturation and fertilization medium (average of 24 oocytes/drop), respectively, with each drop representing one treatment group.

Sperm selection was performed by discontinuous Percoll density gradient centrifugation (same as in Experiment 1) and continuous Percoll density gradient. Four treatment groups were set: TI, TII, TIII and TIV. Continuous gradient was obtained homogenizing the tube contents to mix the 90% and 45% Percoll fractions, resulting in 2 ml of a 67.5% gradient. Gradients were centrifuged at 200 x g. Treatment group distribution is presented in Table 1.

The other steps were performed according to Experiment 1, except that the embryos stayed for 6 days in the foil bag system and morulae and blastocysts were recovered from SOFaaci medium at the end of day 7.

Statistical analysis

Experimental design was of random blocks, with each routine being considered one block. All treatment groups were performed simultaneously and data processed by χ^2 goodness-of-fit test with 5% significance. Male and female percentages obtained in each group were compared with the theoretical percentage of 50% of each gender. Male and female percentages were also compared between treatment groups. Data were processed using SAS statistical program (SAS, 2001).

Table 1. Distribution of treatment groups based on Percoll gradient centrifugation time in Experiment 2.

Treatments	Percoll density gradient	Centrifugation time (min)
TI	Continuous 67.5%	5
TII	Discontinuous 90-45%	5
TIII	Continuous 67.5%	10
TIV	Discontinuous 90-45%	10

Results

In Experiment 1, 327 (65.4%) out of 500 embryos had their gender determined by PCR. From these, 185 (56.6%) were produced by IVF with Percoll (T1) and 142 (43.4%) with swim-up (T2). Male and female embryo percentages obtained in both groups and the comparison between them are presented in Table 2.

In Experiment 2, 345 embryos were produced *in vitro*, from which 329 (95.4%) were amplified. From these embryos, 327 (99.4%) had their gender determined by PCR. Results of the four treatment groups and the comparison among them are presented in Table 3.

Table 2. Percentage of male and female embryos obtained after discontinuous Percoll density gradient centrifugation (90-45%; T1) and swim-up (T2) compared with the expected percentage of 50% of each gender and between treatments. Experiment 1.

Treatments	Embryos n	Male n (%)	Female n (%)	χ^2	P
T1	185	90 (48.65)	95 (51.35)	0.136	0.712
T2	142	83 (58.45)[a]	59 (41.55)[b]	4.056	0.044
Total	327	173 (52.90)	154 (47.09)	1.103	0.294

[a,b]Different superscript letters within rows indicate significant difference ($P < 0.05$).

Table 3. Percentage of male and female embryos obtained after continuous (67.5% for 5 (TI) and 10 (TIII) min) and discontinuous Percoll density gradient (90-45% for 5 (TII) and 10 (TIV) min) compared with the expected percentage of 50% of each gender and among treatments. Experiment 2.

Treatments	Embryos n	Male n (%)	Female n (%)	χ^2	P
TI	93	46 (49.46)	47 (50.54)	0.011	0.917
TII	70	40 (57.14)	30 (42.86)	1.428	0.233
TIII	82	30 (36.59)[a]	52 (63.41)[b]	5.902	0.015
TIV	82	40 (48.78)	42 (51.22)	0.049	0.825
Total	327	156 (47.71)	171 (52.29)	0.688	0.410

[a,b]Different superscript letters within rows indicate significant differences ($P < 0.05$).

Discussion

In Experiment 1, swim-up selected semen resulted in more male embryos (58%) and showed a deviation ($P = 0.044$) from the 50% expected percentage for male and female embryos. As described by Johnson (2000), the Y-chromosome is lighter and smaller than the X-chromosome. The DNA content difference between them is quantified in 3.8%. In addition, McEvoy (1992) believes that Y-bearing sperm is faster than X-bearing sperm. Supporting this study, Pegoraro *et al*. (2002) observed a significant male and female ratio deviation ($P < 0.05$) toward male embryos using swim-up with 45 min incubation. These data suggest that swim-up favors Y-bearing sperm selection, which migrates faster to reach the medium surface. However, in 2006, Rheingantz *et al*. could not repeat the previous results after performing swim-up using 15, 45 and 90 min incubation. Conversely, Madrid-Bury *et al.* (2003), obtained a high percentage of X-bearing sperm performing a double swim-up protocol with the addition of heparin, which reduced sperm motility. They attributed the separation of X- and Y-bearing sperm to occur on the basis of capacitation (acrosome reaction/hyperactivation) rather than motility/viability.

Discontinuous 90-45% Percoll density gradient centrifugation did not show significant difference ($P > 0.05$) on the percentage of male and female embryos (Table 2). This may be explained by the small gradient volume used (2 ml). The mechanism of enrichment of X-bearing spermatozoa by discontinuous Percoll gradients is not fully understood (Kobayashi *et al*., 2004). Even though there is controversy, it is believed that separation occurs as a result of X- and Y-bearing spermatozoa density difference. When Percoll density gradients are prepared in discontinuous fractions, sperm layered on the top gradient (smaller Percoll concentration) naturally penetrate it. The amount of penetration depends on sperm mass and motility. However, when a gradient is centrifuged, the effect of sperm motility is minimized and their mass difference effect is maximized (McEvoy, 1992). It makes the heavier spermatozoa reach the bottom faster; however, the separation threshold between X- and Y-bearing bovine sperm is small because their difference in DNA content is less than 4% (Johnson, 2000). Using flow cytometry, Johnson (2000) observed that in species where the DNA content difference is greater, such as the *Chinchilla langier* (7.5%), a 100% pure selection is possible, but in species where this difference is small, like human beings (2.8%), pureness decreases. In cattle the difference (3.8%) is close to the minimum necessary (3.5%) for separation to occur (Johnson, 2000). The use of larger volume gradients in order to make spermatozoon penetration more difficult could be an alternative. Smaller volumes (1 to 4 ml) may not be enough to promote separation. Larger volumes (>7 ml), however, and higher Percoll concentrations (close to 90%) would turn the swimming down of lighter sperm more difficult. Although male spermatozoa are faster (McEvoy, 1992), their mass is smaller and this effect is maximized during centrifugation. Cesari *et al*. (2006) obtained no difference in the sex ratio of *in vitro* produced bovine embryos using 1.5 ml of a 90-60%-30% discontinuous Percoll density gradient. Supporting the hypothesis of larger volumes, Hossepian-de-Lima *et al*. (2000), using 8 and 12 ml gradients, obtained about 75% females after IVP of bovine embryos with Percoll selected sperm.

Another factor is the speed of sperm penetrating Percoll gradient. Heavier spermatozoa should settle down faster than lighter spermatozoa, therefore centrifugation time could positively influence X-bearing sperm moving down the gradient. The shorter the centrifugation time, the less time the Y-bearing sperm (lighter ones) would have to reach the bottom towards higher Percoll concentration (90%). Centrifugation time used in Experiment 1 was 10 min and probably exceeded the time needed to separate sperm of different mass in the tested gradients.

In Experiment 2, the 67.5% continuous Percoll density gradient centrifugation for 10 min (TIII) showed a deviation ($P = 0.015$; Table 3) in the sex ratio toward female embryos (63%). The other three treatments showed no difference in male and female embryo percentages (Table 3) when compared to the expected 50% of both genders. The same happened when treatments were compared to each other. Continuous Percoll density gradients have already been used to separate or isolate other biological structures (Leuschner *et al*., 1999; Hanabusa *et al*., 2000; Sheoran *et al*., 2005), but have not yet been used to separate X- from Y-bearing sperm. Nevertheless, Woldehiwet *et al*.

(2003) isolated leukocytes from normal and *Anaplasma phagocytophilum* infected sheep attaining high purity (>95%) using 70% and 55% continuous Percoll density gradients, respectively. These data show that continuous Percoll gradients can be explored to separate sperm cell populations of different weights, as it can increase the proportion of X-bearing spermatozoa. Also, further studies with continuous Percoll density gradient combining large volumes and perhaps longer centrifugation time could produce a higher selection of spermatozoa bearing X chromosome.

In conclusion, semen selected by swim-up and 67.5% continuous Percoll density gradient centrifugation for 10 min increased the percentage of *in vitro* produced male and female bovine embryos, respectively.

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