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Assessment of swim-up and discontinuous density gradient in sperm sex preselection for bovine embryo production

[*Avaliação do swim-up e do gradiente descontinuo de densidade na pré-seleção do sexo de espermatozoides na produção de embriões bovinos*]

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ABSTRACT

The purpose of this work was to associate the modified swim-up method with centrifugation in density gradient for the separation of X-bearing spermatozoa. Sperm viability and integrity were evaluated through the Trypan Blue/Giemsa staining method. Quality control of centrifuged spermatozoa was performed in in vitro produced embryos. The results were validated by the sex ratio of in vitro produced embryos using PCR by Y- specific sequences present in bovine male genomic DNA. After determining genetic sex of in vitro produced embryos, the results showed difference ($P<0.05$) in deviation of sex ratio when comparing the control group (45.2% females) with the other spermatozoa selection procedures (60.6% females) ($P<0.05$). The sperm selection methods are capable of selecting X-bearing spermatozoa without compromising the spermatozoa fertility (cleavage and blastocyst rates, 70% and 26%, respectively) and were considered relevant methods to be introduced in bovine in vitro produced embryo programs.

Keywords: density gradient centrifugation, embryo sex ratio, Percoll™, swim-up

RESUMO

O objetivo do presente trabalho foi associar o método de swim-up modificado à centrifugação em gradiente de densidade para a separação de espermatozoides portadores do cromossomo X. A viabilidade e a integridade espermática foram avaliadas pelo método de coloração Azul de Tripan e Giemsa. O controle de qualidade dos espermatozoides centrifugados foi realizado por meio da produção in vitro *de embriões bovinos. Os resultados foram validados pela técnica de PCR para verificar a proporção sexual dos embriões produzidos* in vitro*, com o uso de sequências Y especificas presente no DNA genômico de machos bovinos. Após determinar o sexo genético dos embriões produzidos* in vitro*, os resultados não mostraram diferença (P<0,05) no desvio da proporção do sexo quando comparou o grupo controle (45,2% de fêmeas) com os outros processos de seleção de espermatozoides (60,6% de fêmeas) (P<0,05). Os métodos de seleção de espermatozoides são capazes de selecionar espermatozoides portadores do cromossomo X sem comprometer a fertilidade, medida pelas taxas de clivagem e blastocisto de 70% e 26%, respectivamente, e foram considerados métodos de relevância para serem introduzidos nos programas de produção* in vitro *de embriões bovinos.*

Palavras-chaves: bovino, centrifugação em gradiente de densidade, Percoll™, sexagem de embriões, swim-up

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INTRODUCTION

Methods for sorting bovine sperm into X- and Y-bearing fractions could have a major impact on dairy cattle production, in particular an increase of the reproductive efficiency in breeding programs. Isolation of X-bearing sperm could have important consequences with respect to the availability and affordability of dam selection and heifer replacement (Weigel, 2004; De Vries, 2008), and increase the effectiveness of AI progeny testing programs, as well as the efficiency of multiple ovulation and embryo transfer (MOET) and in vitro embryo production (IVP) programs (Weigel, 2004). In fact, sex pre-selection of implantation embryos, combined with embryo transfer and genetic improvement programs, can increase the profitability of dairy cattle production (De Vries, 2008).

The commercially available technology for separating X- and Y-bearing spermatozoa is flow cytometry with 90% accuracy (Johnson, 2000) and the use of sexed semen technology is the apparent lower fertility of sorted sperm (Palma *et al*., 2008). The sorting procedure might be responsible for variations in cleavage (0 to 89%) and blastocyst rates (3.5 to 28.8%) (Palma *et al*., 2008; Borchersen and Peacock, 2009). There are clear altered mRNA expression patterns of developmentally important genes in *in vitro* produced embryos using sorted semen, showing that sperm sexing by flow cytometry may result in sperm damage, semen fertility reduction (Morton *et al*., 2007) and ongoing pregnancy failure (Perez-Crespo *et al*., 2008). Therefore, other sperm sex selection methods with the purpose of preserving sperm viability have been developed such as the swim-up (Madrid-Burry *et al*., 2003) method and Percoll™ density gradient centrifugation (Hossepian de Lima, 2007).

Percoll™ density gradient centrifugation has been used in humans and cattle to separate sperm cells carrying an X or Y chromosome (Hossepian de Lima, 2007). Selection by Percoll™ discontinuous gradient enhances sperm motility (Parrish *et al*., 1995; Lucio *et al*., 2008), percentage of cells with normal morphology (Prakash *et al*., 1998), intact membrane and intact acrosome (Oliveira *et al*., 2011). Centrifugation of semen *in natura* in density gradient was capable of separating about 70% of X-bearing viable sperm (Hossepian de Lima, 2007) with lower cost and without damages to the sperm acrosome (Resende *et al*., 2010) and plasmatic membrane (Oliveira *et al*., 2011). The swim-up method is based on the difference of swimming speed between X- and Y-bearing spermatozoon (Rodriguez-Martinez *et al*., 1997) and can be used for sperm sex pre-selection (Cesari *et al*., 2006; Yan *et al*., 2006).

The purpose of this work was to evaluate sex ratio deviation of *in vitro* produced embryos using thawed semen in density gradient centrifugation, modified swim-up and modified swim-up associated to density gradient centrifugation.

MATERIALS AND METHODS

All reagents used in this study were obtained from Sigma-Aldrich (St Louis, MO, USA). Percoll™ was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), and BSA was obtained from Calbiochem (Darmstadt, Germany).

Cryopreserved bovine semen was obtained from two Gir Brazilian (*Bos indicus*) and two Jersey bulls (*Bos taurus*), from a commercial company. All sperm samples were evaluated, before and after the treatments, for concentration, total motility and vigor. The percentages of total motile sperm and vigor were determined subjectively, on a drop of semen placed in a pre-warmed glass-slide covered with a coverslip, and examined under a bright-field microscope at 400X magnification. Sperm concentration was determined in a hemocytometer in a 1:200 dilution.

Percoll™ discontinuous gradient was prepared by mixing DMEM isotonic solutions (pH 7.4, 280 to 290mOsm/kg/H_20) with Percoll™ stock solution with 0.3% (wt/vol) BSA, for different densities obtained ranging from 1.110 to 1.123g/mL (Hossepian de Lima, *et al*., 2004). The three layers of discontinuous density gradient were disposed from the larger density (bottom of the tube) to the minor into 15mL conical centrifuge tubes.

For centrifugation, about 40 million frozen-thawed sperm were overlaid on Percoll™ discontinuous density gradient. The gradients were centrifuged at 500 x g for 15 minutes, at

22°C. After centrifugation, the supernatants were removed, the pellets were recovered and sperm motility evaluated. The centrifuged and recovered sperm were used for in vitro fertilization, with a final concentration of 100 x 10^3 spermatozoa/ 20 oocytes.

The modified swim-up was performed according Irianni and Coddington (1992).

The thawed semen samples were deposited in 15mL conical centrifuge tubes containing 5.0mL of DMEM and centrifuged twice at 300 x g for 5 minutes for extender removal. After the second centrifugation the supernatant was discarded and 1.0mL of DMEM medium supplemented with 0.3% BSA was deposited over the pellet, with addition of HEPES. The tube was maintained in an incubator at 38.5ºC for 1h. After this period, 1.0mL of supernatant with approximately 20x10^6 spermatozoa was recovered and sperm motility and vigor were evaluated. The centrifuged and recovered sperm were used for in vitro fertilization, with a final concentration of 100x10^3 spermatozoa/20 oocytes.

About 80 million frozen-thawed sperm were submitted to the modified swim-up. The supernatant was recovered and overlaid on Percoll™ discontinuous density gradient. The gradients were centrifuged and the sperm pellets were recovered. Sperm motility and vigor were evaluated and the recovered and centrifuged sperm were used for in vitro fertilization, with a final concentration of 100x10^3 spermatozoa/20 oocytes.

Sperm membrane and acrossome integrity were assessed using the vital stain Tripan Blue/Giemsa (Sigma-Aldrich St Louis, MO, USA), as described by Kovacs and Foote (1992). An aliquot of semen (20µL) was added to the stain solution (20µL Tripan Blue 0,4%) and incubated at 37°C for 20 minutes. Semen with the stain solution was washed twice in 1mL of distillated water at 700 x g for 5 minutes. The supernatant was discarded and the pellet was resuspended in 0.5mL of distillated water and an aliquot of 10µL was placed on 2 slides and two smears were made from each sample, fixed with methanol for 5 minutes, dried and stained for 18 to 20 hours with Giemsa (10%). For all samples, 100 cells were examined in each slide at 1000 X magnification. The sperm was classified as: live with non-reacted acrosome (LWI), live reacted acrosome (LWA), dead with non-reacted acrosome (DWI) and dead reacted acrosome (DWA).

Bovine ovaries were collected at the slaughterhouse and transported to the laboratory in saline solution at 33°C. Immature cumulus oocyte complexes (COCs) from follicles between 2 and 8mm diameter were aspirated. The COCs were washed in TCM-199 (GIBCO BRL; Grand Island, USA), supplemented with 0.2mM of sodium pyruvate, 20 mM of HEPES, 5mM of sodium bicarbonate, 16.67µg/mL of gentamicin (Sigma-Aldrich St Louis, MO, USA). The oocytes were matured in TCM-199 supplemented with 0.2mM of sodium pyruvate, 25mM of sodium bicarbonate, 16.67µg/mL of gentamicin, 1µg/mL of 17-β estradiol, 0.5µg/mL of FSH (Folltropin™, Bioniche Animal Health, Belleville, EUA), 100 UI/mL of hCG (Profasi HP™; Profasi™, Serono, Sao Paulo, Brazil), plus 10% fetal bovine serum. Twenty COCs per microdroplets were matured in humid atmosphere containing 5% CO_2, for 22 hours, at 38.5°C.

For *in vitro* fertilization (IVF), the matured oocytes were distributed in four groups: control group, modified swim-up, density gradient and modified swim-up associated to density gradient. In the control group, the straws were thawed at 35°C for 30 seconds, and layered on top of a gradient composed of 1mL fraction of 45% and 90% isotonic Percoll™. After centrifugation at 900 x g for 30 minutes, the supernatant was discarded, and the pellet was adjusted with fertilization medium to obtain a final concentration of 100x10^3 spermatozoa in each fertilization droplet. The oocytes and sperm were incubated for 20 hours in 5% CO_2, in humidified air at 38.5ºC, in TALP-IVF medium. Presumptive zygotes were denuded and co-cultured with synthetic oviduct fluid (SOF) medium, and then transferred to 4 well dishes, and embryo culture was carried out at 38.5°C in a humid atmosphere containing 5% CO_2, 5% O_2 and 90% N_2 (Vajta *et al*., 1999). Cleavage was assessed 48 hours after IVF and blastocyst stage on day 7 to 8 of culture. Fetal calf serum (FCS) was not added during in vitro culture, as previously described (Gutiérrez-Ádan *et al*., 2001). Blastocysts were frozen individually into microtubes containing 10µL of double distilled

water and stored at -20°C, for previous determination of the genetic sex by PCR (polymerase chain reaction) analysis.

In order to obtain embryonic cell DNA for sexing by PCR, each embryo was treated with 5µg of proteinase K (Invitrogen, Cleveland, USA), for 60 minutes at 37°C and for 10 minutes at 98°C (for enzyme inactivation).

DNA of each embryo was distributed in two different microtubes (PCR 1 and PCR 2). The PCR 1 primers were: 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' (Bondioli *et al*., 1989) and the second pair was: 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' (Ellis and Harpold, 1986). The PCR 1 detected a sequence of 210bp specific to the bovine Y chromosome and the second pair of primers detected an autosomal sequence of 280bp, indicating the presence of bovine genomic DNA. The PCR 2 primers were: 5'-ATC AGT GCA GGG ACC GAG ATG- 3' and 5'-AAG CAG CCG ATA AAC ACT CCT T-3' (Luz *et al*., 2000). The PCR 2 detected a sequence of 196bp specific to the Y chromosome.

Amplifications were performed as follows: a) for PCR 1: an initial step at 94°C for 10 seconds, 40 denaturation cycles at 94°C for 1 minute, annealing at 58°C for 1 minute and synthesis at 72°C for 1 minute, time extension of 7 minutes at 72ºC was added at the end of the final cycle; b) for PCR 2 primer: initial step at 94°C for 10 seconds, 38 denaturation cycles at 94°C for 1 minute, annealing at 58°C for 1 minute and synthesis at 72°C for 1 minute, time extension of 7 minutes at 72ºC was added at the end of the final cycle as well.

Three repetitions were performed for each group. Sperm viability and acrosome integrity were analyzed with ANOVA, followed by Tukey's test (SAS, 2002). In order to verify the effect of IVP on sex ratio, a control group was established, instead of considering the theoretical ratio of 50:50. The Chi-square test (SAS, 2002) compared the sex ratio of all treatments with the control group and cleavage and blastocyst rates.

For statistical analysis a difference of $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

Increase of sperm viability was observed after all procedures, and the protocols in this study have been used to guarantee sperm viability. After density gradient centrifugation, 4.15% of the total sperm processed were recovered, with an increase of 20% in total motility (80%). Recovery rate after modified swim-up was 18.20%. Alternatively, modified swim-up was associated to the Percoll™ discontinuous density gradient centrifugation with increase in the method's accuracy rate for X-bearing spermatozoa selection. Currently there are no reports in the literature on this association for bovine sperm sex selection.

The data from the Trypan blue-Giemsa stain comparing the spermatozoa classes (LWI, LWA, DWI and DWA) of modified swim-up, Percoll density gradient, modified swim-up associated to Percoll density gradient and control group are in the Table 1.

Table 1. Membrane and acrosomal integrity means of X-bearing sperm selection procedures

Procedure	Classes of spermatozoa			
	LWI	LWA	DWI[1]	DWA
Control	9.25a	52.00a	6.38a	32.38a
Modified swim-up	9.75a	59.25ab	3.25ab	23.88ab
Density gradient	7.00a	71.87bc	1.65b	20.00ab
Modified swim-up associated to density gradient	6.38a	79.37c	0.63b	17.88b
Pr>F	0.2399ns	0.0012s	0.0206s	0.0139s

Within a column, values without a common letter differ ($P<0.05$).
LWI: live with intact acrosome, LWA: live without acrosome, DWI: dead with intact acrosome, DWA: and dead without acrosome
[1]The DWI variable was transformed to $\sqrt{MI + 1}$, for ANOVA to be possible.

Sperm selection procedures induced acrossomal reaction. Modified swim-up associated to density gradient centrifugation selected live spermatozoa but increased acrossomal reaction (79.4%), when compared with all procedures. Watson (2000) described that thawed spermatozoa procedures cause induced sperm plasmatic membrane damages and induce acrosomal reaction. Nevertheless, in order to develop sex selection procedure using thawed sperm it is important to consider the live spermatozoa with non-reacted acrosome, because the acrosome integrity is a sperm characteristic for fertilization (Parrish *et al*., 1988). Treatments to separate X-bearing spermatozoa are able to selected viable sperm but promote acrosomal reaction. Therefore, those spermatozoa can be used in IVP systems, and the induction of sperm capacitation with heparin before oocytes insemination is not necessary (Zhang *et al*., 2003).

No difference in cleavage and blastocyst rates was identified (P>0.05) among sexing groups (modified swim-up, Percoll™ density gradient and modified swim-up associated to density gradient) versus control group (Table 2).

Table 2. Means of cleavage and blastocyst rates of in vitro produced bovine embryos with sperm sexed by modified swim-up, density gradient centrifugation and modified swim-up associated to density gradient centrifugation

Procedure	Oocyte number	Cleavage rate n (%)	Blastocyst rate n (%)
Control	2.015	1.445 (71.71)	522 (25.93)
Modified Swim-up	255	186 (72.94)	83 (32.55)
Density gradient centrifugation	400	282 (70.50)	110 (27.5)
Modified swim-up associated to density gradient centrifugation	363	244 (67.4)	73 (20.17)
Total of oocytes used			3,033

There was no difference between groups (P>0.05) by Chi-square analysis

For in vitro embryo production using sperm selection by Percoll™ discontinuous density gradient centrifugation, the cleavage and blastocyst rates (70.5% and 27.6%, respectively) were similar to the results obtained by Resende *et al*., (2010) using Percoll™ continuous density gradient centrifugation (70% and 24%, respectively). Hossepian de Lima *et al*. (2011) used the same protocol as the present study and observed cleavage and blastocyst rates of 75% and 30%, respectively.

Modified swim-up is a sperm separation procedure frequently used in bovine and human IVP systems to select viable sperm for oocyte fertilization. Using the methodology described by De Jonge *et al*. (1997) for embryo production satisfactory cleavage and blastocyst rates (72.9% and 32.5%, respectively) were obtained.

Difference in sex ratio was identified (P<0.05) among sexing groups (modified swim-up, Percoll™ density gradient and modified swim-up associated to density gradient) versus control group (Table 3).

Table 3. Sex ratio obtained after PCR analysis of in vitro produced bovine embryos with sperm sexed by modified swim-up, density gradient centrifugation and modified swim-up associated to density gradient centrifugation

Procedure	Embryo number	Male n (%)	Female n (%)
Control	427	54.80 (234)	45.20 (193)
Modified swim-up	76	38.20 (29)	61.80 (47)*
Density gradient centrifugation	89	40.60 (36)	59.40 (53)**
Modified swim-up associated to density gradient centrifugation	71	39.40 (28)	60.60 (43) **
Total of embryos sexed by PCR		663	

Difference was observed between control group and the treatments using Chi-square analysis

* P<0.001

**P<0.05

The sexual deviation in favor of females using thawed semen centrifugation in Percoll™ discontinuous density gradient was 59.6% (P<0.05) in agreement to Resende *et al*. (2010) using continuous gradient (60% females). Blottner *et al*. (1993) demonstrated a deviation of 90% in favor of females for IVP embryos. In literature results of fresh sperm sexing using Percoll™ discontinuous density gradient of 55.7% (Kobayashi *et al*., 2004) to 74.3% (Hossepian de Lima, 2007; Hossepian de Lima *et al*., 2011) in favor of females was described.

The deviation towards females could be higher in the present study, whereas control group presented a sex deviation in favor of males (54.80%). In vitro maturation longer than 22 hours (Rizos *et al*., 2008) and the co-incubation oocyte-spermatozoa period (Iwata *et al*., 2007) induced the deviation in favor of males.

The Y-bearing chromosome is faster than the X-bearing chromosome because X sperm has more DNA than Y sperm which results in different migration velocity (Yan *et al*., 2006). Based on this affirmation, some authors described that supernatant of swim-up procedure contained more Y sperm (Check and Katsoff, 1993; Check *et al*., 1994). De Jonge *et al*. (1997), Madrid-Bury *et al*. (2003), Yan *et al*. (2006) and Cesari *et al*. (2006) evaluated the swim-up supernatant and reported no difference between X- and Y-bearing sperm proportion. Bottcher-Luiz *et al*. (1997) observed 69.6% of X-bearing sperm in supernatant. These results were in agreement with the present study (61.80% in favor of females). Rawlings *et al*. (for review see De Jonge *et al*., 1997) reported that after swim-up in the supernatant two fractions could be observed: the lower fraction enriched with Y- bearing spermatozoa (60%) and the upper enriched with X- bearing spermatozoa (64%).

In bovines X-bearing spermatozoa can be separated by discontinuous density gradient centrifugation, modified swim-up and modified swim-up associated with discontinuous density gradient centrifugation with about 60% accuracy. The use of X-bearing spermatozoa is possible in IVP of embryo systems with the purpose of reducing the deviation towards males without compromising embryo development.

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