SEPARATION OF X-BEARING BOVINE SPERM BY CENTRIFUGATION IN CONTINUOUS PERCOLL AND OPTIPREP DENSITY GRADIENT: EFFECT IN SPERM VIABILITY AND IN VITRO EMBRYO PRODUCTION

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ABSTRACT

The aim of this study was to separate X-bearing bovine sperm by continuous Percoll and OptiPrep density gradients and to validate the sexing of resultant in vitro produced embryos by Polimerase Chain Reaction (PCR). Frozen/thawed sperm was layered on density gradients which were previously prepared in polystyrene tubes, 24 h before procedures and maintained at 4 °C. The tubes were centrifuged at 500 x *g* for 15 min at 22 °C. Supernatants were gently aspirated and the sperm recovered from the bottom of the tubes. Viability and integrity of sperm were evaluated by Trypan Blue/Giemsa stain. Cleavage and blastocyst rates were determined by in vitro production of embryos and PCR was performed for identification of the embryos' genetic sex. No damage in viability and acrossomal integrity and in cleavage and blastocyst rates was found in the Percoll and OptiPrep treatment compared to the non-centrifuged group (P>0.05). The percentage of female embryos in the Percoll and OptiPrep group was 63.0 and 47.6%, respectively. The female embryos in control group were 48.7%. A sexual deviation in the Percoll density gradient was achieved without reduction of sperm viability and in vitro production rates.

KEY WORDS: Bovine, centrifugation, *in vitro* production of embryos, PCR, X-bearing sperm.

RESUMO

SEPARAÇÃO DE ESPERMATOZOIDES PORTADORES DO CROMOSSOMO X BOVINO POR CENTRIFUGAÇÃO EM GRADIENTE DE DENSIDADE CONTÍNUO DE PERCOLL E OPTIPREP: EFEITO SOBRE A VIABILIDADE ESPERMÁTICA E NA PRODUÇÃO *IN VITRO* DE EMBRIÕES

O objetivo deste estudo foi separar espermatozoides bovinos portadores do cromossomo X pela centrifugação em gradiente de densidade contínuo de Percoll e OptiPrep, e validar a sexagem pela reação em cadeia da polimerase (PCR), dos embriões produzidos *in vitro*. Para a sexagem, espermatozoides descongelados foram depositados nos gradientes de densidade, previamente preparados, em tubos de poliestireno, 24 horas antes da sexagem e mantidos a 4°C. Centrifugou-se a 500 x *g* por quinze minutos a 22°C. Os sobrenadantes foram aspirados, e os espermatozoides recuperados do fundo dos tubos. Avaliaram-se a viabilidade e a integridade dos espermatozoides pela coloração Azul de Tripan/Giemsa e determinou-se a taxa de clivagem e de blastocisto pela produção *in vitro* dos embriões, identificando-se o sexo genético deste embriões pela PCR. Não foram detectados danos à viabilidade e à integridade acrossomal, nem nas taxas de clivagem e de blastocistos no grupo Percoll e OptiPrep em comparação com o grupo não centrifugado

(P>0,05). A porcentagem de embriões fêmeas no grupo Percoll e OptiPrep foi de 63% e 47,6%, respectivamente, e no grupo controle foi de 48,7%. Houve um desvio na proporção sexual no gradiente de Percoll, sem redução da viabilidade espermática e das taxas de produção *in vitro*.

PALAVRAS-CHAVES: Bovino, centrifugação, espermatozoides X, produção *in vitro* de embriões, PCR.

INTRODUCTION

Sperm sexing rouses great interest due to extensive application in animal production as well as medicine, and new separation techniques which present both better accuracy and low costs are necessary.

A technique which provides accuracy is the flow cytometry that separates, by DNA content, two populations of sperm (X and Y-bearing) with an accuracy of 90% (GARNER, 2006). However this technique has disadvantages such as equipment costs (about US$ 300,000 per machine), damage to sperm during sexing (SEIDEL JR., 2003) and altered mRNA expression of embryos (MORTON et al., 2007).

Using a simple methodology, the density gradient was capable to separate X and Y-bearing sperm with lower cost and without damages to sperm viability. However, this technique presents accuracy of about 70%. In previous studies using the discontinuous Percoll density gradient, HOSSEPIAN DE LIMA et al. (2000) observed many difficulties that prevent commercial application such as the impossibility of gradient storage, that should be used immediately after semen collection or thawing, and moreover difficulties to prepare (many layers) that can increase the variability and it is time consuming. The primary objective of this study was to facilitate the procedure of preparing and storage continuous Percoll and OptiPrep density gradients for separation of X-bearing sperm and to evaluate their efficacy by PCR of embryos in vitro produced.

MATERIAL AND METHODS

The chemicals and media used were purchased from Sigma (Saint Louis, USA), unless otherwise stated.

Semen samples

Cryopreserved bovine semen was obtained from a Brazilian Dairy Gir bull from a commercial company.

Continuous density gradient preparation

Percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden) were made by mixing stock solution of Percoll with DMEM, pH 7.4, 280 to 290 mOsm/Kg/H_20, with 0.3% (wt/vol) of BSA (Calbiochem, Darmstadt, Germany), in order to obtain densities ranging from 1.110 to 1.123 g/mL. OptiPrep was prepared by mixing different proportions in DMEM containing 0.3% (wt/vol) of BSA (Calbiochem, Darmstadt, Germany), pH 7.4 in order to obtain densities ranging from 1.110 to 1.123 g/mL.

Continuous Percoll and OptiPrep density gradients were then consecutively layered in 15 mL polystyrene centrifuge tubes. The tubes were maintained at 4 °C for 24 h to transform a discontinuous density gradient in a continuous density gradient (DENSITY GRADIENT MEDIA, 2005).

For centrifugation, about 30 million sperm were overlaid on Percoll and OptiPrep continuous density gradients. Motility was evaluated subjectively using light microscopy, and concentration by counting spermatozoa in a hemocytometer. They were then centrifuged 500 x *g* for 15 min at 22 to 24 °C. The supernatants were carefully aspirated and the sperm located at the bottom fraction were collected from the tubes. Sperm concentration, motility and recovery rates were determined and the sperm was used for quality control by Trypan Blue/Giemsa stain (assessment of sperm membrane and acrosome integrity) and in vitro production (cleavage and blastocyst rates) procedures.

Assessment of sperm membrane and acrosome integrity

The sperm membrane and acrosome integrity were assessed by light microscopy according to DIDION et al. (1989) with slight modification by the use of the Trypan Blue/Giemsa stain. A sample of 20 µL of each treatment (Percoll and OptiPrep) and a control group (not centrifuged) were incubated with 20 µL of Trypan Blue stain 0.4% at 37 °C for 20 min, and then centrifuged twice at 700 x *g* for 5 min The pellet was re-suspended with 0.5 mL of distilled water, two smears were made from each sample, fixed with methanol for 5 minutes, dried and stained for 18 to 20 h with Giemsa (10%). Slides were evaluated by counting 200 cells in bright field microscope and the sperm was classified as: live with intact acrosome (LWI), live without acrosome (LWA), dead with intact acrosome (DWI) and dead without acrosome (DWA).

In vitro production of bovine embryos

The quality control of the centrifuged sperm was made by evaluation of cleavage and blastocyst rates at days 7 and 8. Bovine ovaries were transported from the abattoir to the laboratory in saline solution at 30 to 35 ºC. Antral follicles of 3 to 7 mm were manually aspirated using a 19-gauge needle attached to a 20 mL syringe. Oocytes with a compact cumulus and at least four layers of cells were selected for in vitro maturation (IVM). Groups of 15 to 20 oocytes were placed in 100 µL droplets of IVM medium under mineral oil. IVM medium was TCM 199 (GIBCO BRL; Grand Island, USA) supplemented with 10% heat-inactivated (at 55 ºC for 30 min) FCS, FSH (1.0 µg/µL; Folltropin™, Bioniche Animal Health, Belleville, EUA), hCG (10 U/mL; Profasi HP™; Profasi™, Serono, Sao Paulo, Brazil), estradiol (1.0 µg/mL), sodium pyruvate (0.2 mM) and 16.67 µg/µL amikacin (Aminocina™, Institute Biochimico, Rio de Janeiro, Brazil) and 5 mM sodium bicarbonate. Oocytes were cultured for 22 h at 38.5 ºC under an atmosphere of 5% CO_2 in the air.

For IVF, control semen (not centrifuged) was thawed at 35ºC for 40 sec. Motile sperm was obtained by centrifugation on a discontinuous Percoll (Amersham Pharmacia Biotech AB; Uppsala, Sweden) density gradient (45%:90%) for 30 min at magnification: x 900. Viable sperm collected at the bottom of the 90% fraction was counted and diluted in IVF medium (100 x 10^3 cells for a 90µL IVF droplet), and incubated for 60 to 90 min for capacitation. The sperm centrifuged in Percoll and OptiPrep density gradients was also counted, diluted and incubated for capacitation before IVF. The oocytes were washed three times in TCM-199 medium (GIBCO BRL; Grand Island, USA) supplemented with 25mM of HEPES, 100mM of sodium pyruvate, BSA (10mg/mL; fraction V, fatty acid free, Inlab, São Paulo, Brazil) and once in IVF medium. The oocytes and sperm were incubated for 20 h in 5% CO_2, in humidified air at 38.5 ºC. Presumptive zygotes were denuded of the cumulus cells by repeated pipetting, and washed three times in modified synthetic oviduct fluid (SOF) medium (VAJTA et al., 1999), and they were then transferred to 500 µL SOF medium in four well dishes. Embryo culture was carried out under mineral oil in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 ºC and assessed the cleavage at 46 to 48 h and development to the blastocyst stage at day 7 to 8. Fetal calf serum (FCS) was not added during in vitro culture, as previously described (GUTIERREZ-ADAN et al., 2001). The blastocysts were frozen in liquid nitrogen, individually, in microtubes with 10 µL of Milli-Q water and stored at -20 °C until the Polimerase Chain Reaction (PCR) analysis for sex determination.

Evaluation of sexing results by PCR analysis

In order to obtain embryonic cell DNA for sex determination by PCR, 176 embryos of the sexing groups (Percoll and OptiPrep) and 78 of the control group were used. Subsequently, Proteinase K (Invitrogen, Cleveland, USA) was added at a final concentration of 5 µg per embryo. The tubes were then incubated at 37 °C for 60 min (for enzyme activity) and at 98 °C for 10 min

(for enzyme inactivation). Two pairs of primers of Y-specific sequences were split in two distinct samples. The first pair was: 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'-ATC AGT GCA GGG ACC GAG ATG- 3' and 5'-AAG CAG CCG ATA AAC ACT CCT T-3' (SCHWERIN et al., 1991; LUZ et al., 2000). The first pair detected a sequence of 210bp specific to the bovine Y chromosome and the second one detected a sequence of 196bp specific to Y chromosome. A third pair of primers detected an autosomal sequence of 280bp, indicating the presence of bovine genomic DNA. The third 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 & HARPOLP, 1986; ELLIS et al., 1988). PCR multiplex was carried out in the same tube with first (210bp) and third (280bp) primers and the PCR of the second pair of primers (196bp) was carried out in another tube. Amplifications were performed in a thermocycler (MJ Research PTC 100 Thermal Cycler, GMI Inc., Minnesota, USA) as follows: a) for first and third primers: an initial step at 94 °C for 10 sec, 40 denaturation cycles at 94 °C for 1 min, annealing at 58 °C for 1 min and synthesis at 72 °C for 1 min. A time extension of 7 min at 72 ºC was added at the end of the final cycle; b) for the second primer: an initial step at 94 °C for 10 sec, 38 denaturation cycles at 94 °C for 1 min, annealing at 58 °C for 1 min and synthesis at 72 °C for 1 min. A time extension of 7 min at 72 ºC was added at the end of the final cycle as well.

Statistical analysis

Three trials were performed for each group. The data of sperm classes from assessment of sperm membrane and acrosome integrity were compared using the Fisher's exact test. Cleavage and blastocyst rates were analyzed by ANOVA, followed by the Tukey's test. To monitor possible interferences in sex ratio during in vitro production a control group (not centrifuged) was established, instead of considering the theoretical ratio of 50:50. The Chi-square test was used to analyze the embryo PCR results, comparing the percentages of male and female embryos of sexing groups (Percoll and OptiPrep) with the control group. For statistical analysis of assessment of sperm membrane ad acrosome integrity and cleavage and blastocyst rates, a difference of $P<0.05$ was considered significant. For PCR results difference of $P<0.10$ was considered significant.

RESULTS

Centrifugation in continuous density gradient

Before centrifugation the mean motility was 70% and after centrifugation, 73% and 77% for Percoll and OptiPrep, respectively. The sperm recovery rate after centrifugation was 4.12% and 16.95% for Percoll and OptiPrep, respectively.

Assessment of sperm membrane and acrosome integrity

The data from the Trypan blue-Giemsa stain (Table 1), comparing the spermatozoa classes (LWI, LWA, DWI and DWA) of Percoll, OptiPrep density gradient and control group. Difference was detected between gradient's groups versus control group in relation to LWI and DWA, improving the quality of sperm after centrifugation ($P < 0.05$).

TABLE 1. *Status* of spermatozoa after Trypan Blue/Giemsa stain of sexing groups (Percoll and OptiPrep) and control group, in according with classes of spermatozoa: live with intact acrosome (LWI), live without acrosome (LWA), dead with acrosome intact (DWI) and dead without acrosome (DWA)

Group	No. of spermatozoa	Classes of spermatozoa (%)			
		LWI	LWA	DWI	DWA
Percoll	200	19[a]	60[a]	6[a]	16[a]
OptiPrep	200	23[a]	59[a]	2[a]	16[a]
Control	200	10[b]	54[a]	2[a]	35[b]

(a and b) Within a column, values without a common superscript differ ($P < 0.05$).

In vitro production of bovine embryos

The findings of in vitro embryo production (cleavage and blastocyst rates) are shown in Table 2. No difference in cleavage and blastocyst rates was identified (P > 0.05) among sexing groups (Percoll e OptiPrep) versus control group.

TABLE 2. Means of cleavage and blastocyst rates of in vitro produced embryos with sperm centrifuged in continuous Percoll and OptiPrep density gradient and control group (not centrifuged)

Group	Oocytes (n)	Cleavage rate n (% ± SD)	Blastocyst rate n (% ± SD)
Percoll	368	260 (70.65 ± 5.29)	87 (23.64 ± 3.57)
OptiPrep	384	290 (75.52 ± 4.51)	106 (27.34 ± 4.14)
Control	332	255 (76.81 ± 5.68)	88 (26.51 ± 2.11)

There were no significant differences among groups (P > 0.05).

Evaluation of sexing results by PCR Analysis

A total of 254 embryos were submitted to genetic sex identification. All embryos were sexed and findings are summarized in Table 3. Data from sexing groups (Percoll and OptiPrep) were compared to the control group. There was sexual deviation for females in the Percoll group (P = 0.10), and not in the OptiPrep group (P = 0.99).

TABLE 3. Sex ratio obtained after PCR analysis of in vitro produced bovine embryos with sperm sexed by centrifugation in continuous Percoll and OptiPrep density gradient, and the control group (not centrifuged)

Group	Total of embryos (n)	Male embryos (% and SD)	Female embryos (% and SD)
Percoll	73	37.0 ± 4.4	63.0 ± 4.4[a]
OptiPrep	103	52.4 ± 8.8	47.6 ± 8.8[b]
Control	78	51.3 ± 5.9	48.7 ± 5.9[b]

In each treatment, the data were accumulated from 3 trials.
Percentages with different superscripts, within columns, differ significantly (P = 0.10).

DISCUSSION

In this study, we used the continuous Percoll and OptiPrep density gradient in attempt to separate X-bearing sperm and use it directly on in vitro fertilization without post-centrifugation washing. Moreover, we verify membrane and acrosome integrity of centrifuged sperm and their consequent influence on bovine in vitro production.

The Trypan Blue/Giemsa stain has been used as an indicative for semen quality evaluation due to its ability to differentiate the live and dead spermatozoa, and to provide the acrosomal status (PARRISH et al., 1988; KÚTVÖLGYI et al., 2006). In another hand, despite the fact that the freezing/thawing procedure causes damages in sperm membrane and loss of acrosome (WATSON, 2000), the stain results demonstrated a difference between gradients versus the control group in relation to spermatozoa with intact acrosome (LWI) and dead without acrosome spermatozoa classes. Regarding the live spermatozoa, LWI are the most important in the fertilization process (PARRISH et al., 1988). However, in the in vitro

production, the live without acrosome spermatozoa (LWA) were the most frequent class identified in this study, it is also able to fertilize, since the centrifugation procedure do not take long time (JOHNSON, 2000).

Our embryo in vitro production results demonstrate that the centrifugation did not interfere in cleavage and blastocyst rates in relation to the control group, indicating that the procedures did not compromise the rates. Despite of sex deviation in the Percoll gradient (63% of females, P = 0.10), the sexing results demonstrated that there was sexual deviation for females after PCR analysis. Results of sexing by discontinuous Percoll gradient made with 12 layers were described in bovines (HOSSEPIAN DE LIMA et al., 2000; KOBAYASHI et al., 2004). In these studies, results ranged from 55.7 to 74.3% of sexual deviation for females. In humans, using the same continuous Percoll density gradient made by 12 layers, there was a variation among 94% (IIZUKA et al., 1987) and 55% for females (WANG et al., 1994). In our study the discontinuous density gradient with only 3 layers, which were transformed to continuous density gradient and could be stored for 24 h at 4 °C, facilitates the routine of in vitro production in specialized companies.

Regarding Optiprep, it has been used in medicine for separation of motile and viable sperm as a substitute of Percoll and was withdrawn since 1996 from clinical human use due to possible endotoxin contamination (McCANN & CHANTLER, 2000; MOUSSET-SIMÉON et al., 2004). The work of MOUSSET-SIMEON et al. (2004) indicated that OptiPrep was less effective than Percoll for separation of sperm with motility and regular morphology. In contrast, we obtained similar results about motility, comparing Percoll and OptiPrep, in agreement with other reports in humans (ANDERSEN & GRINSTED, 1997; HARRISON, 1997; CLAASSENS et al., 1998). However, OptiPrep was not effective for separation of X-bearing sperm.

In conclusion, we applied continuous Percoll e OptiPrep density gradients for separation of X-bearing spermatozoa. The centrifugation procedure did not interfere in the sperm viability as well as on cleavage and blastocyst rates during the in vitro embryo production. There was a sexual deviation in the Percoll gradient.

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