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Comparison of CapriPure® and Percoll® density gradients for sperm separation of frozen-thawed goat spermatozoa

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Abstract

Frozen-thawed semen from six Boer bucks was pooled and used to compare the recently available commercial product CapriPure® with Percoll® for sperm quality. Sperm quality parameters such as sperm motility, concentration, membrane integrity (PI/CFDA), acrosome integrity (FITC-PNA) and mitochondrial activity (JC-1) were evaluated before and after sperm processing using CapriPure® and Percoll® density gradients. There was a significant reduction ($P < 0.05$) in the percentage of spermatozoa with intact acrosomes following Percoll® gradient centrifugation. However, there was a significant increase in the percentage of spermatozoa with high mitochondrial membrane potential in both CapriPure® ($P < 0.01$) and Percoll® ($P < 0.05$) density gradient centrifugation methods. The samples selected using the CapriPure® gradient achieved higher numerical values of membrane integrity, acrosome integrity as well as high mitochondrial membrane potential and showed a tendency for improvement of sperm parameters compared with the Percoll® gradient, suggesting that CapriPure® is a good alternative to Percoll® for goat sperm separation. However, further studies are needed to assess the fertilizing capacity of spermatozoa following the use of the CapriPure® gradient.

Keywords: acrosome, density gradient, mitochondrial activity, sperm preparation.

Introduction

Optimization of assisted reproductive technologies requires the development of rapid, safe, effective methods of selecting functional male gametes through the removal of seminal plasma, cryoprotective agents and cellular debris as well as an increase in the concentration of viable sperm (Rodríguez-Martínez *et al*., 1997). For this reason, sperm separation methods play a very important role.

One method for sperm separation is selective fractionation by density gradient centrifugation. Percoll® is a medium composed of colloidal silica particles (15-30 nm in diameter) coated with polyvinylpyrrolidone (PVP) and is used for the density gradient centrifugation of cells, viruses and subcellular particles as well as for sperm selection (Pertoft, 2000). Numerous studies have demonstrated greater efficiency with the use of Percoll® in sperm preparations (Parrish *et al*., 1995; Rodríguez-Martínez *et al*., 1997). However, Percoll® has not been used in assisted reproductive techniques in human medicine due to its endotoxic effects (Chen and Bongso, 1999).

Therefore, the pharmaceutical industry has developed different media for replacing Percoll®, such as CapriPure®, which is made up of an iso-osmotic salt solution containing colloidal silica particles coated with silane, formulated specifically for use with goat sperm. The number of studies on the effect of Percoll® gradient on frozen-thawed goat sperm is very limited and no studies have been published evaluating CapriPure® gradient or comparing goat sperm obtained from such methods regarding their membrane integrity, acrosome integrity and mitochondrial function.

Therefore, the aim of the present study was to compare the recently available commercial product CapriPure® with Percoll®, based on sperm quality parameters.

Materials and Methods

General approach

For the purposes of our research, a group of six Boer bucks 2-4 years of age with proven fertility was chosen. Semen from four different ejaculates of each buck was collected separately and cryopreserved (skim milk and glycerol 7% in 0.25 ml straws) on four different days. One straw of ejaculate from each buck ($n = 6$) was thawed by immersing it in a 37°C water bath for 30 s then their contents were pooled and sperm parameters were evaluated. Each procedure was repeated four times.

CapriPure® gradient

CapriPure® (Nidacon International AB, Göthenborg, Sweden) was manipulated at room temperature (25°C). In a 15 ml Falcon® tube, 2 ml of CapriPure® Bottom Layer Medium was inserted and then carefully overlayered with 2 ml of CapriPure® Top Layer Medium. Aliquots of 200 µl of thawed semen were placed at the top of the gradient and centrifuged at 300 x *g* for 20 min. The supernatant was removed. The pellet was resuspended in 6 ml of sp-TALP (Tyrode's albumin-lactate-pyruvate medium) and centrifuged At

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500 x *g* for 10 min.

Percoll® gradient

Percoll® gradient was made according to Parrish *et al*. (1995). A 90% isotonic Percoll® solution was prepared by adding 10 ml of a 10 x sp-TALP medium to 90 ml of Percoll® and supplemented with 80 mM NaCl, 3.1 mM KCl, 0.29 mM NaH_2PO_4, 1.97 mM $CaCl_2$, 0.39 mM $MgCl_2$, 10 mM HEPES, 26 mM lactic acid and 25 mM $NaHCO_3$. The 90% Percoll® was mixed with sp-TALP (1:1, v/v) to prepare a 45% Percoll® solution. The Percoll® density gradient was made by layering 2 ml of 45% Percoll® solution on the 2 ml of 90% Percoll® solution in a 15 ml Falcon® tube. On top of the gradient, 200 µl of frozen-thawed semen was layered and then the tubes were centrifuged at 700 x *g* for 15 min. The pellet was resuspended in 6 ml of sp-TALP and centrifuged at 700 x *g* for 5 min. In both procedures, the sperm pellet was resuspended in 200 µl of sp-TALP and subjected to sperm parameters evaluations.

Sperm quality parameters assessment

Sperm quality parameters were evaluated immediately after thawing and after sperm selection methods. Sperm concentration was determined with a haemocytometer. Progressive motility of semen was subjectively assessed by visual estimation under a microscope.

Membrane integrity of spermatozoa was assessed by staining with propidium iodide and 6-carboxyfluorescein diacetate (PI-CFDA) as described by Coleto *et al*. (2002), with modifications. Aliquots of 50 µl of semen were diluted in 150 µl of Tris containing 20 µl of PI (0.5 mg/ml in PBS) and 5 µl of CFDA (0.46 mg/ml in dimethyl sulfoxide - DMSO), incubated at 38°C for 10 min and fixed with PBS containing 0.5% glutaraldehyde. A total of 200 spermatozoa were evaluated under an epifluorescence microscope (Carl Zeiss, Göttingen, Germany) at 400X magnification, using DBP 485/20 nm excitation and DBP 580-630 nm emission filters. Sperm were classified as having an intact membrane when stained green and a damaged membrane when stained red.

For detection of sperm acrossomal integrity, spermatozoa were stained with fluorescein isothiocyanate conjugated to peanut agglutinin (FITC-PNA), following the method described by Roth *et al*. (1998). Aliquots of 5 µl of semen were prepared for smears onto microscope slides and air dried. Twenty microliters of FITC-PNA solution (100 µg/ml) in Phosphate Buffer Saline (PBS) were spread over a smear and incubated in a moist chamber at 4°C for 15 min. The slides were then rinsed twice with refrigerated PBS and dried in the absence of light. Immediately prior to evaluation, 5 µl of the mounting medium (4.5 ml glycerol, 0.5 ml PBS and 5 mg p-phenylenediamine) was placed on the slide and covered with a coverslip. Two hundred spermatozoa were evaluated at 1000X magnification under oil immersion using BP 450-490 nm excitation and LP 515 nm emission filters. Spermatozoa were then classified as having intact acrosomes when the acrosome region stained with green fluorescence or as reacted acrosomes when a fluorescent green band was found in the equatorial region of the sperm head or there was no green fluorescence in the entire head region.

Mitochondrial function was determined by lipophilic cationic JC-1 (Guthrie and Welch, 2006). Aliquots of 50 µl of semen were diluted in 150 µl of Tris containing 5 µl of JC-1 (0.15 mM in DMSO), incubated at 38°C for 10 min and fixed with PBS containing 0.5% glutaraldehyde. A total of 200 spermatozoa were evaluated at 1000X magnification under oil immersion using a BP 450-490 nm excitation and LP 515 nm emission filters. Cells stained orange were classified as having high mitochondrial membrane potential, whereas cells stained green were classified as having low membrane potential.

Statistical analyses

Differences among treatments were evaluated using analysis of variance (ANOVA) following arcsin transformation (arcsin $\sqrt{P/100}$) of the percent values and Tukey's multiple-comparison test, using the INSTAT program for Windows (version 3.01).

Results

No significant differences ($P > 0.05$) were found in sperm concentration, progressive motility and membrane integrity among the semen samples evaluated immediately after thawing and following the use of the Percoll® and CapriPure® density gradient methods. Results are shown in Table 1.

Table 1. Mean (±SD) characteristics of frozen-thawed goat sperm immediately after thawing and following selection using the Percoll® and CapriPure® density gradient methods.

Treatment	Concentration (x 10^6/ ml)	PM (%)	MI (%)	AcI (%)	hMMP (%)
Post-Thaw	135.0 ± 46.6	53.8 ± 7.5	41.9 ± 9.1	66.9 ± 16.2[a]	10.8 ± 12.8[a]
Percoll®	47.0 ± 39.0	61.2 ± 7.5	31.2 ± 3.0	36.5 ± 8.3[b]	49.1 ± 10.1[b]
CapriPure®	59.5 ± 53.0	55.0 ± 5.8	40.6 ± 19.1	42.6 ± 15.2[ab]	63.2 ± 12.9[b]

PM= progressive motility; MI= membrane integrity; AcI= acrosome integrity; hMMP= high mitochondrial membrane potential. Within a column, values without a common superscript differ ($P < 0.05$).

There was a significant reduction ($P < 0.05$) in the percentage of spermatozoa with intact acrosomes following Percoll® gradient centrifugation when compared to the percentage in the sample immediately after thawing, whereas no significant difference was found ($P > 0.05$) between the sample immediately after thawing and the sperm selected using the CapriPure® gradient.

A significant difference was found in the percentage of spermatozoa with high mitochondrial membrane potential between the samples evaluated immediately after thawing when compared to the samples submitted to selection using the Percoll® ($P < 0.05$) and CapriPure® ($P < 0.01$) density gradient methods. However, no significant difference ($P > 0.05$) was found in high mitochondrial membrane potential between the samples selected using the Percoll® and CapriPure® density gradient methods.

Discussion

In all the semen samples analyzed in the present study, the percentage of spermatozoa with intact membranes was lower than the percentage of motile spermatozoa. Cryopreservation is known to cause damage to the plasma membrane and to the integrity of the organelles by altering the organization of the lipid bilayer as well as through the peroxidation of the membrane lipids (Silva and Gadella, 2006). Thus, it is possible that some motile spermatozoa in the present study had membranes damaged by the freezing-thawing process. On the other hand, this result may have been limited by the use of subjective motility analysis, resulting in erroneous motility estimates as suggested by Vertegen *et al*. (2002).

Studies have evidenced that the centrifugation force can affect motility and the membrane integrity of sheep (Gil *et al*., 1999) and goat (Ritar, 1993) spermatozoa. These reports indicate that methods involving mechanical stress, such as centrifugation, are harmful to the viability of goat sperm. Due to this fact, and the different centrifugation speeds used in each gradient method, the results of the sperm parameters obtained with the two methods should be interpreted with caution.

Sperm parameters in the samples obtained after the CapriPure® method proved similar to those submitted to the Percoll® gradient. The use of silane-coated silica particles (BoviPure®) for density gradient centrifugation of frozen-thawed bull sperm revealed no significant differences ($P > 0.05$) in motility, concentration, membrane integrity or acrosome integrity when compared to the Percoll® gradient (Samardzija *et al*., 2006).

Also, in cryopreserved bovine sperm, the preparation in the PureSperm® gradient has not been found to influence overall motility, but has significantly improved the proportion of spermatozoa with intact acrosomes. That beneficial effect using this gradient only occurred when the samples exhibited low post-thaw motility (Maxwell *et al*., 2007). The PureSperm® and BoviPure® gradients are an iso-osmotic salt solution containing colloidal silica particles coated with silane and differ very little from the CapriPure® protocol.

The significant reduction in the percentage of spermatozoa with intact acrosomes when using the Percoll® gradient are in disagreement with results from previous studies that reported a greater percentage of sperm cells with intact acrosome membranes after using this density gradient on cryopreserved goat semen (Rho *et al*., 2001) and bovine semen (Somfai *et al*., 2002). The lower percentage of cells with intact acrosomes after the use of the Percoll® gradient in the present study likely occurred due to the capacitation phenomenon and acrosome reaction induced by this selection method. In sheep semen, a greater percentage of capacitated spermatozoa have been found using the Percoll® density gradient method when compared to samples of fresh semen (Marti *et al*., 2006).

The PVP in the Percoll® has adverse effects on plasma, acrosome and mitochondrial membranes in human spermatozoa (Strehler *et al*., 1998). There are reports claiming that different batches of Percoll® differ in composition (Avery and Greve, 1995). However, throughout the entire experiment, the density gradient was prepared with Percoll® from a single batch.

Mitochondrial status plays an important role due to its relation with the energy profile, sperm motility and, consequently, fertility (Kasai *et al*., 2002). The freezing-thawing process of spermatozoa obtained from Murciano-Granadina goats has been found to considerably reduce the number of cells with high mitochondrial membrane potential (Marco-Jiménez *et al*., 2006). In the present study, there was a significant increase in the percentage of spermatozoa with high mitochondrial membrane potential in both density gradient centrifugation methods CapriPure® ($P < 0.01$) and Percoll® ($P < 0.05$) compared to the values obtained immediately after thawing, thereby revealing efficiency in the selection of viable gametes, regardless of the gradient used.

The samples selected using the CapriPure® gradient achieved higher numerical values of membrane integrity, acrosome integrity as well as high mitochondrial membrane potential and showed a tendency for improving sperm parameters compared to with the Percoll® gradient, suggesting that CapriPure® is a good alternative to Percoll® for buck sperm separation. However, further studies are needed to assess the fertilizing capacity of these spermatozoa following the use of the CapriPure® gradient.

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References

Avery B, Greve T. 1995. Impact of Percoll on bovine spermatozoa used for in vitro insemination. *Theriogenology*, 44:871-878.

Chen MJ, Bongso A. 1999. Comparative evaluation of two density gradient preparations for sperm separation for medically assisted conception. *Hum Reprod,* 14:759-764.

Coleto ZF, Guerra MMP, Batista AM. 2002. Avaliação do sêmen congelado de caprinos com drogas fluorescentes. *Rev Bras Med Vet*, 24:101-104.

Gil J, Söderquist L, Rodríguez-Martínez H. 1999. Influence of centrifugation and different extenders on post-thaw sperm quality of ram sperm. *Theriogenology,* 54:93-108.

Guthrie HD, Welch GR. 2006. Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. *J Anim Sci*, 84:2089-2100.

Kasai T, Ogawa K, Mizuno K, Nagai S, Uchida Y, Ohta S, Fujie M, Suzuki K, Hirata S, Hoshi K. 2002. Relationship between sperm mitochondrial membrane potential, sperm motility and fertility potential. *Asian J Androl*, 4:97-103.

Marco-Jiménez F, Viudes-de-Castro MP, Balasch S, Mocé E, Silvestre MA, Gomez EA, Vicente JS. 2006. Morphometric changes in goat sperm heads induced by cryopreservation. *Cryobiology*, 52:295-304.

Martí E, Pérez-Pé R, Muiño-Blanco T, Cebrián-Pérez JA. 2006. Comparative study of four different sperm washing methods using apoptotic markers in ram spermatozoa. *J Androl,* 27:746-753.

Maxwell WMC, Parrilla I, Caballero I, Garcia E, Roca J, Martinez EA, Vazquez JM, Rath D. 2007. Retained functional integrity of bull spermatozoa after double freezing and thawing using PureSperm® density gradient centrifugation. *Reprod Domest Anim*, 42:489-494.

Parrish JJ, Krogenaes A, Susko-Parrish JL. 1995. Effect of bovine sperm separation by either Swim-up or Percoll method on success of in vitro fertilization and early embryonic development. Theriogenology, 44:859-869.

Pertoft H. 2000. Fractionation of cells and subcellular particles with Percoll. *J Biochem Biophys Methods*, 44:1-30.

Rho GJ, Hahnel AC, Betteridge KJ. 2001. Comparisons of oocyte maturation times and of three methods of sperm preparation for their effects in the production of goat embryo in vitro. *Theriogenology*, 56:503-516.

Ritar AJ. 1993. Control of ovulation, storage of semen and artificial insemination of fibre-producing goats in Australia: a review. *Aust J Exp Agric,* 33:807-820.

Rodríguez-Martínez H, Larsson B, Pertoft H. 1997. Evaluation of sperm damage and techniques for sperm clean up. *Reprod Fertil Dev*, 9:297-308.

Roth TL, Weiss RB, Buff JL, Bush LM, Wildt DE, Bush M. 1998. Heterologous in vitro fertilization and sperm capacitation in an endangered African antelope, the Scimitar-Horned Oryx (Oryx dammah). *Biol Reprod*, 58:475-482.

Samardzija M, Karadjole M, Matkovic M, Cergolj M, Getz I, Dobranic T, Tomaskovic A, Petric J, Surina J, Grizelj J, Karadjole T. 2006. A comparison of BoviPure® and Percoll® on bull sperm separation protocols for IVF. *Anim Reprod Sci*, 91:237-247.

Silva PFN, Gadella BM. 2006. Detection of damage in mammalian sperm cells. *Theriogenology*, 65:958-978.

Somfai T, Bodó S, Nagy S, Papp AB, Iváncsics J, Baranyai B, Gócza E, Kovács A. 2002. Effect of Swim-up and Percoll treatment on viability and acrossome integrity of frozen-thawed bull spermatozoa. *Reprod Domest Anim*, 37:285-290.

Strehler E, Baccetti B, Sterzik K, Capitani S, Collodel G, De Santo M, Gambera L, Piomboni P. 1998. Detrimental effects of polyvinylpyrrolidone on the ultrastructure of spermatozoa (Notulae Seminologicae 13). *Hum Reprod*, 13:120-123.

Verstegen J, Iguer-Ouada M, Onclin K. 2002. Computer assisted semen analyzers in andrology research and veterinary practice. *Theriogenology*, 57:149-179.