Isolate® and Optiprep® minigradients as alternatives for sperm selection in bovine in vitro embryo production

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Vianna, L. L., Pradieé, J., Santos, E. C. S., Gonçalves, A. O., Pfeifer, L. F. M., Rheingantz, M. G. T., Dode, M. A. N., Vieira, A. D., Lima, V. F. H., Correa, M. N. and Pegoraro, L. M. C. 2014. **Isolate® and Optiprep® minigradients as alternatives for sperm selection in bovine in vitro embryo production**. Can. J. Anim. Sci. **94**: 35–42. The objective of this study was to evaluate alternatives in small volumes to conventional gradient of Percoll® on semen quality, in vitro embryo production, sex ratio and embryo survival after vitrification. Thawed semen was randomly allocated to one of four density gradient selection methods: (1) conventional Percoll® (P), (2) MiniPercoll (MP), (3) MiniIsolate (MI), and (4) MiniOptiprep (MO). Sperm kinetics and quality were evaluated. Use of P, MP and MI gradients did not affect sperm motility ($P > 0.05$). However, there was a decrease in total and progressive sperm motility in MO (70.8 and 51.3% vs. 87.3 and 69.5% for P; 87.3 and 73% for MP; 92.3 and 78.8% for MI; $P < 0.05$). The MO had lower membrane integrity compared with P, MP and MI (39.7 vs. 70.5, 72.3, 63.8%, respectively, $P < 0.05$). The percentage of blastocysts produced was higher in MI than in MP and MO (21.1 vs. 16.1 and 16.9%, $P < 0.05$) and similar to P (18.4%; $P > 0.05$). Sex ratio and embryo survival after vitrification were similar among groups ($P > 0.05$). Semen selected by Isolate and Optiprep gradient, at the concentrations and small volumes used, demonstrated similar characteristics and in vitro embryo production to conventional Percoll® gradient.

Key words: In vitro fertilization, sperm selection, sex ratio, sperm quality, cryopreservation

Vianna, L. L., Pradieé, J., Santos, E. C. S., Gonçalves, A. O., Pfeifer, L. F. M., Rheingantz, M. G. T., Dode, M. A. N., Vieira, A. D., Lima, V. F. H., Correa, M. N. et Pegoraro, L. M. C. 2014. **Mini-gradients Isolate® et Optiprep® comme choix alternatifs pour la sélection des semences dans la production in vitro d'embryons bovins**. Can. J. Anim. Sci. **94**: 35–42. L'objectif de cette étude était d'évaluer les choix alternatifs aux gradients traditionnels Percoll®, en petits volumes, sur la qualité de la semence, la production in vitro d'embryons, le rapport des sexes et la survie de l'embryon après vitrification. Des semences décongelées ont été attribuées aléatoirement à une de quatre méthodes de sélection par gradient de densité : (1) Percoll® traditionnel (P), (2) MiniPercoll (MP), (3) MiniIsolate (MI), et (4) MiniOptiprep (MO). La cinétique et la qualité des spermatozoïdes ont été évaluées. L'utilisation des gradients P, MP et MI n'ont eu aucun effet sur la motilité des spermatozoïdes ($P > 0{,}05$). Il y avait, cependant, une diminution des motilités totales et progressives dans le gradient MO (70,8 et 51,3 % c. 87,3 et 69,5 % pour P; 87,3 et 73 % pour MP; 92,3 et 78,8 % pour MI; $P < 0{,}05$). Le gradient MO avait une intégrité de membrane inférieure aux gradients P, MP et MI (39,7 c. 70,5; 72,3; 63,8 % respectivement, $P < 0{,}05$). Le pourcentage de blastocystes produits était plus élevé après gradient MI qu'après les gradients MP et MO (21,1 c. 16,1 et 16,9 %, $P < 0{,}05$) et similaire après gradient P (18,4 %; $P > 0{,}05$). Le rapport des sexes et la survie de l'embryon après vitrification étaient similaires parmi tous les groupes ($P > 0{,}05$). Les semences choisies par gradients Isolate et Optiprep, aux concentrations et aux petits volumes utilisés, démontrent des caractéristiques et une production in vitro d'embryons similaires à celles choisies par gradients Percoll® traditionnels.

Mots clés: FIV, sélection des semences, rapport des sexes, qualité du sperme, cryopréservation

Under in vivo conditions, potentially fertile spermatozoa are selected during migration through the female reproductive tract. However, when assisted reproductive techniques are performed, it is necessary to artificially select motile spermatozoa (Cesari et al. 2006). Semen selection procedures are routinely applied to prepare semen for in vitro fertilization. Semen selection is used to separate viable from non-viable sperm and to remove seminal plasma, cryoprotectants, as well as other background material, debris, and infectious agents (Henkel

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Abbreviations: **COC**, cumulus oocytes complexes; **FDA**, 6-carboxyfluorescein diacetate; **IVP**, embryo production; **MI**, MiniIsolate; **MO**, MiniOptiprep; **MP**, MiniPercoll; **P**, Percoll®; **PVP**, polyvinylpyrrolidone

and Schill 2003). A Percoll® density gradient is the most common method used to select sperm for embryo production (IVP) (Cesari et al. 2006). However, toxic effects of the Percoll® gradient on sperm and embryos have been described (De Vos et al. 1997); these were attributed to polyvinylpyrrolidone (PVP). Therefore, although the use of Percoll® in human-assisted reproduction is prohibited worldwide (McCann and Chantler 2000), and its use in animals is being challenged, it is still a method of choice for animal assisted reproductive techniques. Hence, alternative protocols are needed. Moreover, protocols that reduce the time needed for preparation of semen and/or the volume of the gradient would be ideal.

Isolate® and PureSperm® gradients, both of which contain colloidal silica, represent alternative methods for sperm selection. There is also Optiprep®, which contains iodaxanol (Harrison 1997; Claassens et al. 1998; Mendes et al. 2003; Mousset-Siméon et al. 2004; Resende et al. 2009). When Optiprep® was used in a conventional gradient, there was no damage in viability and acrossomal integrity and blastocyst rates (27.34 ± 4.14 for Optiprep® versus 23.64 ± 3.57 for Percoll® conventional) (Resende et al. 2009). The Isolate® gradient is used more often in human reproductive techniques, with good results (Mousset-Siméon et al. 2004), although its influence on sex ratio has apparently not been reported.

With the purpose of developing alternatives to conventional Percoll®, the objectives of the present study were to evaluate the effects of Percoll®, Isolate® and Optiprep® density in small volumes gradients on: (1) sperm quality, (2) embryo development, (3) sex ratio, and (4) embryo viability after cryopreservation of bovine embryos produced in vitro.

MATERIALS AND METHODS

Chemicals

Media used for in vitro embryo production, maturation medium, fertilization medium, sperm wash medium (Sp-TALP), gradient Percoll 90% and synthetic oviduct fluid (SOFaa) medium were purchased from Nutricell® (Campinas, SP, Brazil). Isolate® was purchased from Irvine Scientific (Santa Ana, CA), and Optiprep® was purchased from Sigma (St. Louis, MO).

Sperm Selection Procedures

Gradient Preparation and Treatments

Three gradients were divided into the following four groups: (1) Percoll® (P) and (2) MiniPercoll (MP), which were colloidal silica covered with PVP (90% Percoll); (3) MiniIsolate (MI), a sterile colloidal silica solution stabilized with covalent ligations of hydrophilic silane (90% Isolate®), and; (4) MiniOptiprep (MO), composed of iodixanol (60% Optiprep®).

For P, 2 mL of 90% Percoll® was placed in a 15-mL polystyrene tube followed by the same amount of 45% Percoll®. For MP, 400 μL of 90% Percoll®, followed by the same amount of 45% Percoll®, were placed in a 1.5-mL microtube. For MI, 400 μL of 90% Isolate® and then 400 μL of 45% Isolate® were placed in a 1.5 mL microtube. Finally, in MO, a small volume gradient was performed in three concentrations of 30, 28 and 26% of Optiprep®. In a 1.5-mL microtube 400 μL of each layer were added. All reagents were diluted in Sp-TALP media. After gradients were prepared, they were stabilized for 60 min in an incubator (39°C and 5% CO_2).

For the entire experiment, semen from the same ejaculate of a Jersey bull was used. Just before fertilization, semen was thawed at 35°C for 1 min. Then, 300 μL of semen was added to the surface of each gradient and centrifuged as follows: P, $700 \times g$ for 20 min; MP, $700 \times g$ for 5 min; MI, $700 \times g$ for 5 min; and MO, $900 \times g$ for 15 min. Following the first centrifugation, supernatants were discarded. Pellets containing sperm were re-suspended in 2 mL (P) or 400 μL (MP, MI and MO) of Sp-TALP, and centrifuged at $700 \times g$ for 5 min. The resulting pellets were re-suspended in 40 μL of fertilization medium.

Sperm Quality Parameters Assessment

Semen parameters were assessed before and after density gradient centrifugation.

An aliquot (10 μL) of semen was placed on a slide (Leja standard count, SC20.01.04.B, 20 μM, Leja Product B. V., the Netherlands) to evaluate sperm kinetics using a computer-assisted semen analysis system (Analysis System, Ivos-Ultimate 12's, Hamilton Thorne Biosciences, Beverly, MA). End points analyzed included: total motility (TM), progressive motility (PM), track velocity (VAP), linear speed (VSL), curvilinear velocity (VCL), lateral amplitude of the head (ALH), tail beat frequency (BCF), and linearity (LIN). The microscope was adjusted according to the manufacturer's instructions for evaluation of bovine semen and four visual fields were selected manually for analysis.

Sperm membrane integrity was assessed using 6-carboxyfluorescein diacetate (FDA) combined with propidium iodide (PI; Molecular Probes®, Eugene, OR) as described (Harrison 1997). The semen sample (10 μL) was added to 30 μL of staining solution, which was composed of 5 μL formol saline (96 mL of 0.9% saline and 4 mL of 40% formol) 5 μL of PI (0.75 mM), 10 μL FDA (0.46 mg mL^{-1} in DMSO), and 480 μL of 3% sodium citrate. For each slide, 200 sperm were counted, using a filter for wavelengths 494/517 nM excitation/emission, at $1000 \times$ magnification in immersion oil. Green (FDA)-stained cells were considered to have an intact membrane, whereas those stained red (with PI) were considered to have a damaged membrane (Fig. 1).

Acrosome integrity was evaluated with the fluorescent probes isothiocyanate-conjugated peanut agglutinin (FITcC-PNA) and PI, as described by Klinc and Rath (2007). An aliquot (10 μL) of semen was diluted in 30 μL

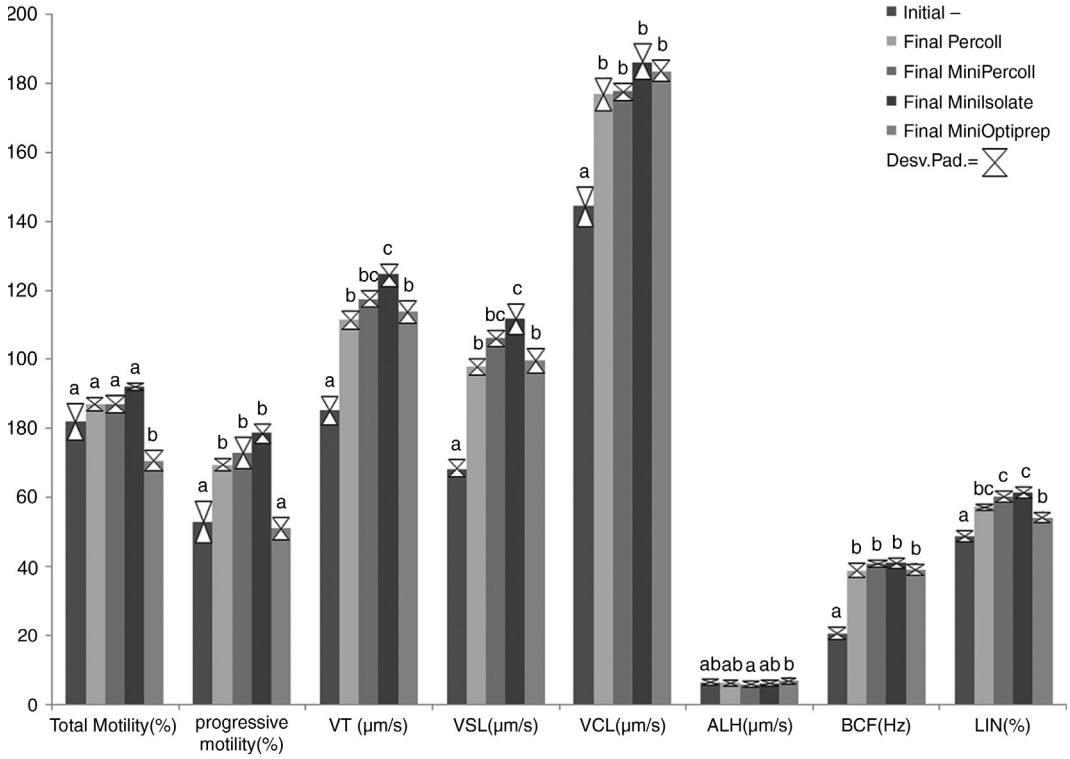

Fig. 1. Sperm kinetic before (control) and after going through the gradients (Mean ± SEM).
a–c Within a column, means without a common superscript differ ($P < 0.05$). VT, track velocity; VSL, linear velocity; VCL, curvilinear velocity; ALH, lateral amplitude of the head; BCF, tail beating frequency; LIN, linearity.

of stain solution, containing 5 µL formol saline (96 mL of 0.9% saline and 4 mL of 40% formol = formalin), 5 µL of PI (0.75 mM), 10 µL FITC – PNA (0.46 mg mL^{-1} in PBS), and 480 µL of 3% sodium citrate. Two hundred cells were counted with a filter for wavelengths 497/517 nM excitation/emission, at 1000 × magnification (with immersion oil). Sperm were initially evaluated using bright-field microscopy and subsequently using fluorescence microscopy; those visualized under bright field, but not visible under fluorescence, and those with an intact acrosome, were considered alive. Sperm visualized under fluorescence were classified as alive with a reacted acrosome (acrosomal region colored green, FITC – PNA), dead with an intact acrosome (colored red, PI), or dead with a reacted acrosome (colored red with a green acrosomal region green, PI/ FITC – PNA).

Semen evaluations were carried out in the Animal Reproduction Laboratory of Embrapa Genetic Resources and Biotechnology (Cenargen, Brasília, DF, Brazil). Four replicates were done to evaluate sperm kinetics, whereas three replicates were done to evaluate acrosomal and membrane integrity.

In Vitro Embryo Production

Bovine ovaries (*Bos taurus*) were obtained at the slaughterhouse and within 2 h were transported (30°C) to the laboratory in 0.9% saline solution supplemented with 50 µg mL^{-1} gentamicin sulfate. Follicles, 2 to 8 mm in diameter, were aspirated using a vacuum pump (80 mmHg; digital vacuum pump WTA®). Oocytes were washed in wash media, and only Cumulus oocytes complexes (COCs) with a homogenous cytoplasm and several layers of cumulus cells were used. The COCs selected for in vitro embryo production were divided into groups of 30 to 35 and placed in 400-µL wells of maturation media covered with silicone oil and incubated for 22 h at 39°C with 5% CO_2 and saturated humidity. After 22 to 24 h of in vitro maturation, COCs were transferred directly into wells containing 400 µL of fertilization medium. The COCs were divided into groups of 30 to 35, which were randomly assigned to one of the treatments: P ($n = 830$), MP ($n = 897$), MI ($n = 648$), and MO ($n = 780$). Following sperm selection and motility evaluation, sperm concentration was measured using a Neubauer chamber. The insemination dose was 1×10^6 motile sperm mL^{-1} of medium. After insemination, sperm and COCs were incubated for 18 to 22 h at 39°C and 5% CO_2, with saturated humidity.

Fertilization was designated day 0. On day 1, presumptive zygotes were denuded by multiple pipetting, washed in SOFaa medium, and placed in culture in 400-µL drops, with 30 to 35 structures per well.

Cleavage rates were evaluated 48 h post-insemination, and embryo development rates were recorded on days 7 (morulae and blastocyts/oocytes inseminated) and 8 (blastocysts/oocytes inseminated). Zygotes/embryos were cultured for 8 d in SOFaa media (Takahashi and First 1992), supplemented with 5% FCS and covered with silicone oil and incubated at 39°C and 5% CO_2, with saturated humidity.

Embryo Sexing

Expanded day 8 blastocysts from each group (P = 116, MP = 103, MI = 81, and MO = 115) were selected for sex determination. The sex of embryos was determined with PCR. Two sequences of known DNA were used, one specific for an autosomal bovine gene (Invitrogen, Cleveland, OH), 216 bp long, and the other for a specific region of the Y chromosome (Invitrogen), 175 bp long. After evaluation of embryo development on day 8, embryos were washed in PBS containing 2% PVA and stored individually in microtubes with 20 μL of lysis solution [1% proteinase K (20 mg mL^{-1})], 1% 10 × PCR buffer, and 8% ultrapure water). Embryos with a zona pellucida were placed in a pronase solution (0.25%) for degradation and then washed and stored. To determine sex, embryos stored in the lysis solution were incubated for 60 min at 37°C for cellular lysis and then incubated at 95°C for 15 min to inactivate proteinase K. Embryo sex was determined as described (Rheingantz et al. 2006). Products amplified by PCR were separated in a 2.5% agarose gel; after electrophoresis, the gel was placed in an ethidium bromide bath, and DNA fragments were visualized under a UV light. If there was only one DNA band (216 bp), the embryo was designated as female, whereas those with two bands (216 and 175 bp) were considered male. There were also four controls: a negative control (no DNA, to exclude contamination), a female control (DNA from a bovine female), a male control (DNA from a bovine male), and a 100 bp ladder (Ludwig Biotec, Porto Alegre, RS, Brazil).

Embryo Vitrification

To assess blastocyst quality, Grades I or II expanded blastocysts were vitrified using an open pulled straw (OPS) method (Vajta et al. 1998). Vitrification was performed in two steps: (1) embryos were placed in vitrification solution 1 (VS1: 1.05 M DMSO, 1.34 M ethylene glycol in TCM 199 with 20% FCS) for 3 min and subsequently; (2) transferred to vitrification solution 2 (VS2: 2.1 M DMSO, 2.68 M ethylene glycol in TCM with 20% FCS and 0.3 M sucrose) for up to 40 s. Then, embryos were loaded in the OPS, immersed in liquid nitrogen, and stored in a cryogenic container. Equilibration in the cryoprotectant solution and warming were done on a thermal plate (39°C). Warming and embryo rehydration used stepwise sucrose gradients (0.6 and 0.3 M for 5 min each in TCM with 10% FCS). After rehydration, embryos from all groups (P, $n = 47$; MP, $n = 42$; MI, $n = 37$; and MO, $n = 43$) were subjected to 48 h of additional IVC in 100 μL drops of embryo-conditioned SOFaa media. Survival was based rates of re-expansion and hatching after incubation for 24 and 48 h, respectively.

Statistical Analyses

The data were analyzed using the SAS program (SAS Institute, Inc., version 8.0, Cary, NC). Chi-square was used to analyze binomial variables (e.g., cleavage rate on day 2, embryo development on days 7 and 8, survival and cryopreservation after 24 and 48 h, and the male:female ratio). Production was expressed as a percentage of embryos produced (days 7 and 8) per total matured oocytes. Semen characteristics, such as acrosome and membrane integrity, and sperm kinetics were analyzed by ANOVA. Tukey-test was used to compare treatment means (relative to an evaluation performed immediately after thawing, i.e., Initial). All statistical analyses were performed at 5% probability.

RESULTS

Semen characteristics before (initial analyses) and after gradient treatments (P, MP, MI, and MO) are shown (Fig. 1). Sperm selected with MO had the lowest motility ($P < 0.05$). Moreover, MO and initial analyses had lower progressive motility than other groups ($P < 0.05$). Compared with initial analyses, all treatments improved VT, VSL, VCL, BCF, and LIN ($P < 0.05$). No difference in acrosome integrity was detected between initial analyses and treatment groups ($P > 0.05$, Fig. 2). However, the MO had lower membrane integrity compared with P, MP and MI (39.7 vs. 70.5, 72.3, 63,8%, respectively, $P < 0.05$; Fig. 2.

Cleavage rates and embryo development are shown (Fig. 3). Semen obtained by Percoll density gradient resulted in higher cleavage rate than MP and MO ($P < 0.05$). MO had the lowest cleavage rate among groups ($P < 0.05$). In contrast, semen selected with MI density gradient produced higher percentage of day 7 embryos ($P < 0.05$). Comparable results were detected on the percentage of day 8 embryos; P and MI had similar embryo production percentage ($P > 0.05$).

From the 415 day 8 embryos selected for sex determination, 411 were successfully sexed by PCR. The sex ratios obtained in all treatments were not different from the expected 1:1 ratio ($P > 0.05$). The sex ratios (male:female) detected were 48:67, 42:60, 30:50, and 57:57, for P, MP, MI and MO, respectively.

The percentage of re-expanded embryos at 24 h after warming, was higher in MP group (92.8%) than other groups; 72.3, 64.8, and 69.7%, for P, MI and MO, respectively; $P < 0.05$). However, when hatching was evaluated at 48 h after warming, no difference was detected among groups (42.5% P, 52.2% MP, 37.8%, MI; and 39.5% MO; $P > 0.05$).

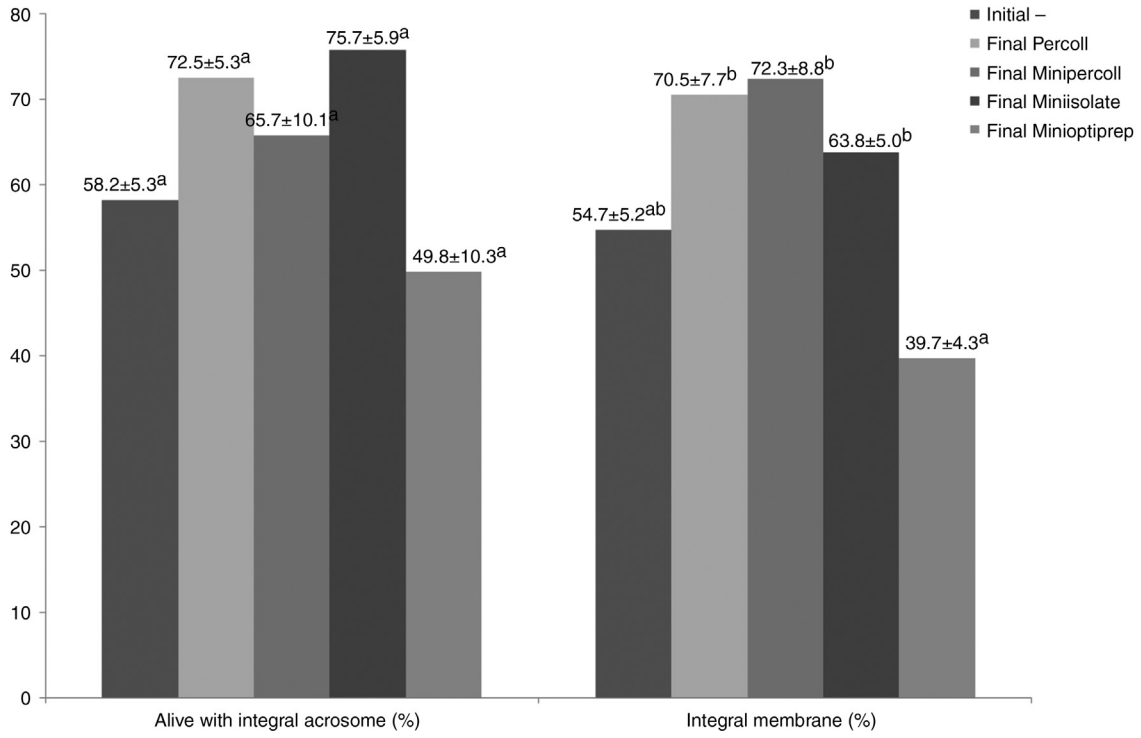

Fig. 2. Integrity of the acrosome and membrane before control) and after running through the gradients (Mean ± SEM). a–b Within a column, means without a common superscript differ ($P < 0.05$).

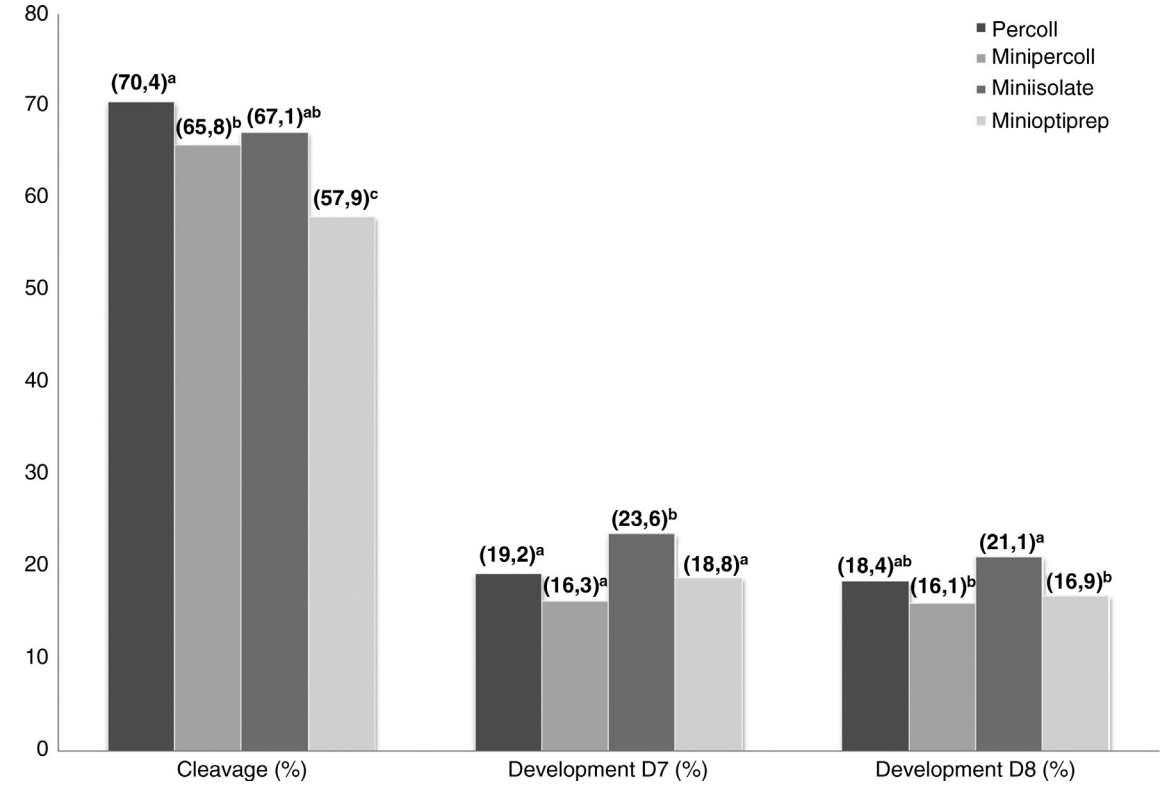

Fig. 3. Cleavage and embryo development rate on D7 and D8. a–c Within a column, means without a common superscript differ ($P < 0.05$).

DISCUSSION

In this study, to compare the efficiency and quality of the different gradients used to selected semen, variables directly associated to sperm quality were evaluated. Furthermore, data regarding sperm capability and embryo quality, production and survival, were also accessed to provide data to prove that the best semen would provide higher embryo development rates and quality. Although no differences in embryo production was detected among groups at final evaluation in day 8, an 800-μL minigradient, centrifuged at $700 \times g$ for 5 min demonstrate to be an alternative to conventional Percoll. Therefore, the use of minigradients for sperm selection could be of great interest for in vitro fertilization laboratories, as it would reduce cost and preparation time.

Many groups have reported that Percoll® gradient is the preferred method to prepare sperm for in vitro fertilization. It is recognized as one of the best methods for separating spermatozoa (McCann and Chantler 2000; Petyim et al. 2009). However, it has been reported that PVP has deleterious effects on the spermatozoa (Strehler et al. 1998) and the embryo (De Vos et al. 1997). Hence, Percoll® is no longer used in clinical practice for human-assisted reproductive technology (McCann and Chantler 2000). Moreover, centrifugation is a potentially sperm-damaging step in sperm processing (Aitken and Clarkson 1988) and Percoll® passage imposes mechanical contact with the cells. It has been reported that passage through a Percoll® gradient can alter the plasma membrane and cause a premature acrosome reaction (Cesari et al. 2006), due to membrane destabilization. However, these alterations were not detected among treatments in the present study. In that regard, the data demonstrate that minigradients yielded as many embryos as Percoll® gradient, suggesting that they are appropriate alternatives to Percoll® for sperm selection in IVP procedure. Therefore, we inferred that the gradients did not induce a premature acrosomal reaction. In contrast, Cesari et al. (2006) reported that Percoll® significantly decreased the proportion of sperm with an intact acrosome. The inconsistent results may be due to diverse concentrations and volumes of the Percoll® gradients used in various studies (Oliveira et al. 2010). Nevertheless, we believe that this is more related to the gradient concentrations, because in our study, there was no significant difference for various volumes, i.e., 4.0, 1.2 and 0.8 mL for P, MO, and MP and MI, respectively.

It has been reported that sperm motility (Parrish et al. 1995), percentage of cells with normal morphology (Prakash et al. 1998), intact membrane (Tanghe et al. 2002) and intact acrosome (Somfai et al. 2002; Cesari et al. 2006) were enhanced after Percoll® selection. However, no differences were detected in sperm kynetic and integrity of the acrosome and membrane in all treatment tested in this study. Many authors suggest that the sperm progressive motility is improved after Percoll centrifugation (Cesari et al. 2006; Lee et al. 2009; Machado et al. 2009; Mehmood et al. 2009; Petyim et al. 2009). Similarly, progressive motility was improved, not only for P, but also for MP and MI. The absence of a significant difference in total motility following selection with gradients compared with unselected sperm was attributed to the excellent semen quality (post-thaw total motility was $82.3 \pm 5.5\%$). One may correctly contest the use of only one bull throughout the treatments in the study. However, in this very first study we aimed to investigate the potential use of minigradients in IVP procedure. Although the present experiment was not designed to address the possible variations among different bulls, the results demonstrate exciting data that provides background before starts further and deeper studies using several bulls. Therefore, further studies are necessary to elucidate if minigradients can be used to select semen for IVP routine.

It has been stated that the Percoll® centrifugation method is effective in selecting motile cells with intact plasma membrane and high mitochondrial membrane potential. However, it caused damage to the acrosomal membrane (Oliveira et al. 2011). In contrast, in this study, acrosome integrity was not different between treatments and initial evaluation. Normally, sperm with intact membranes and acrosomes has good mitochondrial function, and consequently, good fertilizing capacity (Flesh and Gabella 2000; Celeghini et al. 2008). In that regard, sperm selected with MO had fewer cells with intact membranes, therefore, it could have negatively affect the cleavage rates in MO group.

When we compared embryo yield among groups, apparently the Miniisolate group tended to develop more embryos. It is noteworthy that although both Isolate® and Percoll® are formed by a colloidal silica, the latter contains PVP, whereas the former does not. Moreover, the concentration and volume used in the MI and MP were the same. Therefore, we speculate that the difference in the embryo production may have been due to the toxicity of the PVP present in the Percoll® (De Vos et al. 1997; Strehler et al. 1998; Kato and Nagao 2009). The use of a conventional Isolate® gradient was recently reported for bovine in vitro embryo production using sexed and unsexed sperm (Rodríguez Villamil et al. 2012). In that study, Isolate® was a more effective method for the recovery of high-quality sperm and also yielded more embryos than the Percoll® gradient. Although Isolate® is frequently used for human-assisted reproduction, presumably the high cost limits its use in other species. However, protocols with reduced volumes, such as the one used in the present study, could make it a cost-effective option.

The difference in DNA mass between X and Y chromosome-bearing sperm causes a difference in weight, and density may promote their separation by density gradients (Parrilla et al. 2004; Machado et al. 2009). It is well known that X and Y sperm have differences in size and DNA content, and consequently a

difference in weight and density, which may promote their separation by density gradients. However, all four gradients preserved the expected 50:50 (male:female) ratio, consistent with previous studies using Percoll (Cesari et al. 2006; Machado et al. 2009). Although other studies have reported a difference in the sex ratio depending on the stage of development, since females develop slower than males (Rheingantz et al. 2006), no such difference was proved in the present study.

Resistance to cryopreservation was used to evaluate embryo quality, since those with better quality are more resistant to this process. In the present study, despite a higher re-expansion rate for MP than the other treatments, hatching rates were all similar.

CONCLUSION

The data from this study show that the use of small volume gradients did not negatively affect the sperm quality and embryo yield when compared with a conventional Percoll® gradient. Furthermore, centrifugation at $700 \times g$ for 5 min was shown to be an alternative to Percoll®. Finally, the Isolate® and the Optiprep® gradients at the concentration and small volume used in the present study were viable alternatives to the Percoll® gradient for in vitro produced bovine embryos.

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