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Contributed Paper

Influence of Discontinuous PureSperm® and OptiPrep™ Gradient Centrifugations on Bovine Sperm Quality and the Sex Ratio of *in vitro* Produced Embryos

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ABSTRACT

Presently, separation of X- and Y-sperm with flow cytometry is only one successful method of sex selection; however, well-trained personnel and costly instrument are needed for this technique. Therefore, separation with a more simple and convenient method (i.e. a gradient centrifugation) becomes of interest. Influence of 8-layer PureSperm® and OptiPrep™ density gradients on the quality of bovine sperm and the sex ratio of *in vitro* produced embryos was evaluated. Fresh semen (n = 12) with the sperm motility of at least 65% was divided into four aliquots. One aliquot served as a non-centrifuged control sample was frozen in Tris-egg yolk extender. The other three were applied to 8-layer gradients in PureSperm®, OptiPrep™ or Percoll. After centrifugation, the sperm pellet was added with the extender and then frozen. The thawed semen was evaluated for the sperm quality; the sex ratio of sperm was determined in the *in vitro* produced embryos by multiplex PCR. The viability, acrosome morphology and membrane integrity (HOST) of thawed sperm in the PureSperm® and Percoll groups were similar to the control ($p > 0.05$) and were significantly higher than those in the OptiPrep™ ($p < 0.0001$ to $p = 0.03$). The PureSperm®, OptiPrep™ and Percoll centrifugations did not show a significant increase in X-bearing sperm in the pellet (61.6%, 61.0% and 54.3%, respectively) compared to the control sample (58.8%, $p > 0.05$). In conclusions, centrifugation of fresh bovine semen in discontinuous 8-layer PureSperm® gradients did not damage the survival of frozen-thawed sperm. However on the basis of testing in the *in vitro* produced embryos by multiplex PCR, discontinuous PureSperm® and OptiPrep™ gradient centrifugations were not able to deviate the sex ratio of bovine sperm.

Keywords: dairy cattle, centrifugation, X-bearing sperm, *in vitro* fertilization, polymerase chain reaction

1. Introduction

Artificial insemination (AI) is an insemination technique which has been most widely used for propagating cattle population especially dairy cattle [1]. In theory, if fertilization and conception succeed following insemination with non-sex-sorted sperm, the ratio of getting male to female offspring is approximately 1:1 (50%:50%) [2]. This seems to be unwanted by dairy cattle producers whose businesses focus on milk production. Therefore, sex selection of sperm prior to conception (called "sperm sexing") is very useful in the dairy cattle industry, to produce the optimal proportion of males and females to take advantage of sex-influenced traits and thus to permit higher productivity [3].

Methods of separating X- from Y-bearing sperm are composed of, for example, discontinuous albumin gradient centrifugation [4], Sephadex gel filtration [5], Percoll gradient centrifugation [6], swim-up procedures [7], and flow cytometry [8]. Unfortunately, none of the mentioned methods except flow cytometry have met with success sustainable and reproducible enough to achieve differentiation of X- and Y-bearing sperm [9]. Presently, the use of Percoll, which is a silica-based colloidal medium and the silica particles of the medium are coated with polyvinylpyrrolidone (PVP), is likely to be inhibited especially in human assisted reproductive technologies (human ART) due to uncertain efficiency, reports of endotoxin contamination, variation in composition between batches, and occurrence of inflammatory responses in female reproductive tissues after insemination with Percoll treated sperm [10,11]. In spite of being the only scientifically proven method of sex selection, flow cytometric separation of X- and Y-chromosome bearing sperm is nowadays available only in some highly developed countries such as UK and USA [3]. It is due to that this technique needs well-trained personnel to conduct measurement, by using a costly advanced instrument (i.e. flow cytometer). Therefore, separation of X- and Y-bearing sperm by means of a gradient centrifugation method, which is more simple and convenient than flow cytometry, using an alternative gradient solution such as PureSperm® (Nidacon, Gothenburg, Sweden) and iodixanol instead of Percoll become of interest.

PureSperm®, a sterile colloidal silica suspension in an isotonic salt solution, was actually designed to use in human sperm to alleviate the potential problems associated with using Percoll [12]. The advantages of PureSperm® over other sperm selection gradients such as Percoll have also been reported in isolating ram [13], and bovine [14] sperm, with improved viability, membrane integrity and DNA integrity. Iodixanol, originally developed as an X-ray contrast agent, is another substance nowadays widely applied as a medium for density gradient centrifugation to isolate viable cells [15]. This substance has been proven to be safety for using in human due to its low endotoxin level [16]. Using 60% iodixanol in water prepared commercially as OptiPrep™ (Axis-Shield, Oslo, Norway) to select sperm with good quality in human and bovine, the comparable results about motility were investigated in samples processed with Percoll and OptiPrep™ [16, 17]. However, there has been only one report on using OptiPrep™ to select X-bearing bovine sperm and unsatisfied results were revealed when OptiPrep was prepared as continuous density gradients [17]. To our knowledge, the effectiveness of PureSperm® in separating X from Y sperm (sperm sexing) has never been tested in any species including bovine. Also, discontinuous OptiPrep™ gradients have never been applied to sort the sex of bovine sperm. This study was therefore designed to apply the methods of discontinuous PureSperm® and OptiPrep™ density gradients for sex pre-selection in fresh bovine sperm. Successes of the techniques were evaluated in resultant *in vitro* produced bovine embryos using the polymerase chain reaction (PCR).

2. METHODS

All media components used in this study, which were indicated as substance names (no. xxx, Lot/Batch yyy), were obtained from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated.

2.1 Animals

At "Farm Chokchai" in Nakhon Ratchasima province, four mature 50 to 100% Holstein-Friesian bulls were selected to include in this study. These bulls were good in fertility profiles and were being used as semen donors for production of frozen-thawed semen.

2.2 Semen Collection

Semen was collected once a week via an artificial vagina from each bull (n=3 ejaculates/bull). Only raw semen with a minimum of 65 % individual progressive motility was further processed by being split into four fractions: (I) the control, semen was frozen without being sorted, as ordinarily conducted; (II) PureSperm, semen was destined for sorting by a discontinuous PureSperm® gradient centrifugation; (III) OptiPrep, semen was sorted by OptiPrep™ density gradients; and (IV) Percoll, X- and Y-bearing sperm in the semen were separated using a previously developed technique, discontinuous Percoll gradients.

2.3 Preparation of Density Gradients

2.3.1 Discontinuous pureSperm® gradients

Various concentrations of PureSperm® (i.e. 40, 50, 60, 70, 75, 80, 85, and 90%) was prepared by diluting PureSperm® 100 (Nidacon, Sweden) with the modified Tyrode's medium [1X sperm-TALP; 2 mM $CaCl_2$ (no. C-7902, Lot 44H01385), 3.1 mM KCl (no. P-5405, Lot 34H03025), 0.4 mM $MgCl_2$ (no. M-2393, Lot 54H03085), 100 mM NaCl (no. S-5886, Batch 066K01282), 25 mM $NaHCO_3$ (no. S-5761, Batch 113K0025), 0.3 mM NaH_2PO_4 (no. S-9638, Lot 18H0957), 1 mM Na-pyruvate (no. P-5280, Lot 064K06183), 21.6 mM Na-lactate (no. L-4263, Lot B124K5303), 10 mM HEPES (no. H-6147, Lot 091M54252V), 6 mg/mL BSA (fraction V) (no. A-9647, Batch 087K0747), and 50 μg/mL gentamycin (A.N.B. Laboratories Co., Ltd., Bangkok, Thailand)] (pH=7.4) [18]. Discontinuous 8-step PureSperm® gradients were prepared in 15 mL centrifuge tubes by consecutively layering 1 mL each of 90% (bottom) to 40% (top) PureSperm® solutions.

2.3.2 Discontinuous optiprep™ gradients

In group III, 10, 15, 20, 25, 30, 35, 40, and 45% OptiPrep™ were provided by diluting OptiPrep™ (60% iodixanol, density 1.32 g/mL; Axis-Shield, Norway) with 1X sperm-TALP. An 8-step OptiPrep™ gradient was made in 15 mL centrifuge tubes by consecutively layering 1 mL each of 45 (bottom), 40, 35, 30, 25, 20, 15, and 10 (top) % OptiPrep™ solutions.

2.3.3 Discontinuous percoll gradients

A discontinuous Percoll gradient centrifugation was used, as a reference centrifugation method of sperm sex selection, to separate X- from Y-bearing sperm in samples assigned to group IV. A 90%

isotonic Percoll solution was obtained by mixing 9-volume of Percoll™ (GE Healthcare Bio-sciences AB, Uppsala, Sweden) with 1-volume of 10X sperm-TALP. The 90% Percoll was further mixed with 1X sperm-TALP at different ratios to make 40, 50, 60, 70, 75, 80, and 85% Percoll solutions. Preparation of an 8-layer Percoll gradient column in a 15 mL tube was conducted in the same manner as described for PureSperm®, but the Percoll solutions were used instead.

2.4 Separation of X- and Y-bearing Sperm

Discontinuous PureSperm®, OptiPrep™, and Percoll gradient centrifugations were used to separate X- from Y-bearing sperm in samples assigned to group II, III, and IV, respectively. The separation protocol was modified from the procedure originally designed for Percoll [6] and described briefly as follows.

Collected fresh semen containing the sperm motility of ≥ 65% was further evaluated its quality in the aspects of the sperm concentration, sperm viability, acrosome morphology, and plasma membrane integrity. The qualified fresh semen assigned to groups II, III, and IV was diluted with 1X sperm-TALP to a concentration of approximately 400×10^6 sperm/mL. The diluted semen (1 mL each) was placed as the topmost layer of the discontinuous density gradients prepared previously. Following centrifugation at 500 X g for 20 min and removal of supernatant, the remaining pellets in the bottom of the tubes suspected as samples enriched with X-bearing sperm were washed in 1X sperm-TALP (sperm : extender = 1:4, v:v) by centrifuging at 300 X g for 10 min. The washed sperm pellets (0.5 mL each) were further frozen.

2.5 Sperm Freezing and Thawing

The untreated control sample (I) and the sorted sperm (II, III, and IV) were frozen with Tris-egg yolk extender using the freezing protocol modified from Anzar et al. [19]. Briefly, 1 mL of the samples was extended at room temperature in 3 mL of Tris-egg yolk extender [3.0 g Tris (Research Organics, Inc., Ohio, USA), 1.7 g citric acid (no. C-2404, Batch 115K00951), 1.3 g fructose (no. F-0127, Lot 125H0567), 0.1 g penicillin-streptomycin (no. S-9137, Lot 025K0630), 8% (v:v) glycerol (no. G-9012, Batch 034K0011) and 20% (v:v) egg yolk]. The diluted samples were gradually cooled to 4°C and further kept at this temperature for 4 h. At 4°C, mini-straws (0.25 mL) were filled with the processed sperm, frozen using a styrofoam box and stored in the liquid nitrogen tank for at least 1 d until thawing. The straws were thawed in water at 37°C for 30 s.

2.6 Evaluation of Fresh and/or Frozen Thawed Semen Quality

2.6.1 Semen volume

The volume of fresh semen was measured in mL using a collecting tube at the time of collection.

2.6.2 Sperm concentration

The concentration of each ejaculate was determined with a photometer (SpermaCue™, Minitüb GmbH, Tiefenbach, Germany).

2.6.3 Sperm motility

The individual progressive motility of sperm was assessed subjectively by the same technician using a bright field microscope (40X).

2.6.4 The sperm viability and acrosome morphology

These two parameters were evaluated independently under a light microscope (1000X) following eosin-nigrosin staining [20]. A total of 200 sperm was evaluated per attribute per sample. Sperm with an unstained head were regarded as the live sperm; sperm with a crescent shaped apical ridge were considered as the morphologically normal acrosome sperm. An example of sperm stained with eosin-nigrosin dye is depicted in Figure 1.

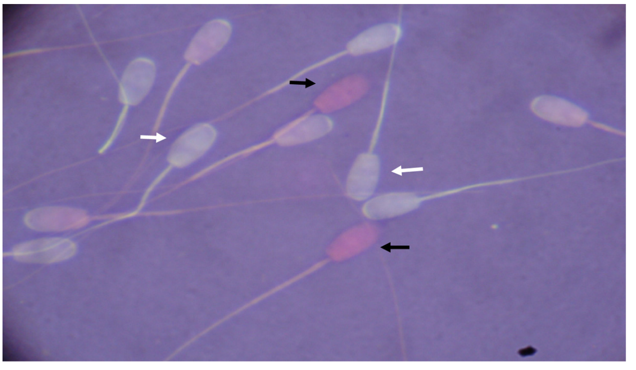

Figure 1. The viability of sperm was evaluated under a light microscope (1000X) following eosin-nigrosin staining. Sperm with an unstained head (white arrows) were regarded as the live sperm. Sperm having red head (black arrows) were deemed as dead sperm.

2.6.5 Sperm plasma membrane integrity

The integrity of sperm plasma membrane was assessed using the hypo-osmotic swelling test (HOST) [21]. In each sample, the percentage of sperm with intact membrane was assessed from 200 total cells under a microscope (1000X) after being incubated at 37°C for 15 min with the hypo-osmotic solution [fructose and Na-citrate (no. W-302600) in distilled water; 75 mOsm/kg], fixed in the hypo-osmotic solution plus 5% formaldehyde (Merck, Germany) and placed on a glass slide with a cover slip. Sperm expressing coiled tail were determined as sperm with plasma membrane intact (see in Figure 2).

Figure 2. The integrity of sperm plasma membrane was assessed using the hypo-osmotic swelling test (HOST). Sperm expressing coiled tail (arrows) were determined as sperm with plasma membrane intact.

2.7 Identification of X-bearing Sperm

The degree of enrichment of X-bearing sperm in the non-sex-sorted (I) and sex-sorted (II, III, and IV) frozen-thawed sperm was determined indirectly in embryos. The embryos were produced *in vitro*, using procedures modified from Pozzobon et al. [22], after oocyte fertilization with frozen-thawed sperm in groups I to IV. The embryos were tested to find out their sex by using multiplex PCR [23]. The *in vitro* production (IVP) of embryos and subsequent sexing by PCR were conducted in the laboratory of the Institute of Molecular Biosciences (MB), Mahidol University, Salaya.

2.8 *In vitro* Embryo Production

Follicles with the diameter of 2 to 8 mm were aspirated from bovine ovaries that were previously collected from a slaughterhouse and transported to the laboratory within 2 h after collection in 0.9% NaCl added with 40 mg/mL gentamycin at 37°C. Follicular fluid was also transferred to Petri dishes for oocyte classification under a stereo microscope. Only oocytes with quality of 1 or 2, i.e oocytes with a compact multilayered cumulus investment and homogenous ooplasm, were washed and transferred into a dish containing the maturation medium. The maturation medium was composed of tissue culture medium 199 (TCM 199; Gibco™, NY, USA) supplemented with 10% (v:v) fetal bovine serum (FBS; no. F-1051), 0.2 mM Na-pyruvate, 5 mg/mL LH (no. L-9773, Lot 21K1356), 0.5 mg/mL FSH (no. F-4021), 1 mg/mL estradiol 17b (no. E-2758, Lot 85H10535), and 40 mg/mL gentamycin. The oocytes were matured *in vitro* by incubating at 39°C in 5% of CO_2 in air with high humidity for approximately 20 h. After the observation of cumulus oophorus expansion, the oocytes were washed and transferred into drops (approximately 10 oocytes/drop) of Fert-TALP medium with 3 mg/mL BSA, 0.11 mg/mL Na-pyruvate, 30 mg/mL heparin (Leo, Leo Pharmaceutical Products, Ballerup, Denmark), 10 mM caffeine (no. C-0750, Batch 028K0757), and gentamycin under mineral oil. For IVF, the frozen bull semen (groups I to IV) was thawed and then selected for vigorously motile sperm using the swim-up technique. Oocytes (approximately 40 oocytes/group) were inseminated with the selected sperm (1×10^6/ml of medium); the co-culture (sperm + oocytes) was incubated at 39°C in 5% CO_2 in air and saturated humidity for approximately 20 h. Following IVF, oocytes and zygotes were denuded, washed, and transferred into drops of modified synthetic oviduct fluid (mSOF) medium [107.7 mM NaCl, 7.16 mM KCl, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.78 mM $CaCl_2$, 1.51 mM $MgSO_4$, 7.27 mM Na-Pyruvate, 5.35 mM Na-lactate, 0.2 mM L-Glutamine (no. G-8540, Lot 082K0362), 2.8 mM myo-inositol (no. I-7508, Lot 021M0135V), 0.3 mM Na-citrate, 45 mg/mL BME amino acid 50X (no. B-6766, Lot RNBC0637), 5 mg/mL MEM amino acid 100X (no. M-7145, Lot RNBC0314), 5% FBS, and 50 mg/mL gentamycin; modified from Holm et al. [24]]. Zygotes were incubated in Petri dishes under mineral oil in 5% CO_2 in air at 39°C and saturated humidity for 7 d. The developed embryos (≥2-cell stage) were determined their sex by multiplex PCR [23].

2.9 Multiplex Polymerase Chain Reaction

Single bovine embryos placed in 6 μL DNAse/RNAse-free distilled water were extracted DNA through lysing at 95°C for 10 min. The sex of embryos was identified by multiplex PCR using simultaneous analyses of the Y-specific and fragment of bovine satellite chromosome, according to the procedure explained by Rattanasuk et al. [23] with some modifications. In brief, the amplification reactions were conducted in a total volume of 20 μL including 10X PCR buffer, 50 mM $MgCl_2$, 10 mM dNTPs, 5 μM BSP [the bovine-specific primers: BSP_f (Batch SG00057557) and BSP_r (Batch SG00057558)], 10 μM BY [the bovine Y-specific primers: BY_f (Batch SG00057559) and BY_r (Batch SG00057560)], 1 U Taq DNA polymerase and 2 μL of the lysed products used as a DNA template. A DNA thermal cycler (Palm Cycler; Cybeles Life Science, Postfach, Heidelberg, Germany) was used to amplify the DNA with the first denaturation step at 95°C for 2 min, 45 cycles of 95°C for 20 s, annealing at 52°C for 45 s, and extension at 72°C for 50 s followed by final extension at 72°C for 10 min. In negative control reaction, no DNA template was used. DNA isolated from muscle tissue of bull and cow was used in case of positive control. PCR products (8 μL) were electrophoresed on 2% agarose gel (Vivantis Technologies Sdn. Bhd., Selangor Darul Ehsan, Malaysia), then stained with ethidium bromide (no. E-2515, Lot 35H0868), and finally visualized under an UV illuminator (BioDoc-lt™ System, CA, USA).

2.10 Statistical Analysis

Statistical Package for the Social Sciences (SPSS Statistics 17.0, Chicaco, IL) was used for statistical anlyses. The sperm parameters were presented as mean ± Standard Error of the Mean (SEM). Differences in the percentages of sperm motility, viability, acrosome morphology, plasma membrane integrity, and the cleavage rates of embryos among groups were analysed with one-way ANOVA. When ANOVA showed a significant effect, values were compared using the Least Significant Difference (LSD) test. The X:Y ratios among samples in groups I, II, III, and IV were analysed using Pearson's chi-squared test. The level of significance was defined as $p \leq 0.05$.

3. Results

On average of twelve samples collected, the semen volume and the sperm concentration were 4.7±0.4 mL and 1.9±0.1 $\times 10^9$ sperm/mL, respectively. The average percentages of motility, viability, acrosome morphology, and plasma membrane integrity (HOST) of sperm both in fresh collected and frozen-thawed samples, together with the percentages of sperm recovered after centrifugation (the sperm recovery rates) are demonstrated in Table 1.

Table 1. Fresh and frozen-thawed sperm quality in non-sex-sorted (I) and sex-sorted (II, III, and IV) samples (n = 12).

Parameters (%)	Fresh	Frozen-thawed			
		I) Control	II) PureSperm	III) OptiPrep	IV) Percoll
Recovery rate[1]	-	-	61.9±4.2	46.6±4.2	86.5±3.3
Motility	68.3±1.1	53.3±3.0^{a}	42.1±2.4^{bc}	37.1±2.9^{c}	45.0±2.1^{b}
Viability	87.0±2.0	70.8±2.1^{a}	66.8±2.9^{a}	46.1±3.4^{b}	66.1±3.7^{a}
NAR[2]	86.2±1.4	69.0±2.1^{a}	62.4±3.6^{a}	47.3±3.2^{b}	67.7±1.7^{a}
HOST[3]	74.7±2.8	58.2±2.8^{a}	51.6±3.1^{a}	41.7±2.6^{b}	53.2±3.4^{a}

[1]Recovery rate = (sperm concentration after centrifugation x 100)/sperm concentration before centrifugation. [2]NAR = normal acrosome morphology. [3]HOST = sperm with plasma membrane intact. Value (mean±SEM) with different superscripts (^{a,b,c}) indicate significant difference within rows ($p \leq 0.05$).

Irrespective of types of the gradient media tested, the post-centrifuged sperm recovery rates were ranged between 46.6 to 86.5%. All sperm parameters, except for the sperm motility, evaluated after thawing in the PureSperm® (II) and Percoll (IV) groups were not different from those of the control (I) ($p > 0.05$). On the other hand, the quality of frozen-thawed sperm previously separated with OptiPrep™ (III) was lower ($p < 0.001$) than that of non-sex-separated sperm (I).

The efficiency of the gradient media in separation of X-bearing sperm was evaluated indirectly through *in vitro* produced embryos. It has been found that the cleavage rate of embryos in the centrifuged samples was not different from that of the non-centrifuged sample (79.5±3.9%, 80.0±4.1%, 78.4±4.1% and 79.9±3.8% for I, II, III, and IV, respectively; $p > 0.05$). However regarding the embryos' sex ratio, none of the gradient media (II, III, and IV) successfully improved the proportion of female embryos compared with the control sample (I) ($p > 0.05$). The sex ratios of *in vitro* produced embryos are shown in Table 2.

Table 2. Percentage of male and female obtained after PCR analysis of *in vitro* produced bovine embryos with sex-sorted sperm (II, III, and IV), compared separately with non-sex-sorted sperm (I).

Groups	Total number of embryos	Number of embryos (%)	
		Male	Female
I) Control	404	167 (41.2)	237 (58.8)
II) PureSperm	399	153 (38.4)	246 (61.6)
III) OptiPrep	386	151 (39.0)	235 (61.0)
IV) Percoll	381	174 (45.7)	207 (54.3)
Total	1570	-	-

There were no significant differences ($p > 0.05$) among data (%) within columns.

4. Discussion

In this study, processes of X-sperm separation using 8-layer PureSperm® and Percoll gradients yielded the similar frozen-thawed sperm quality, and most of the sperm quality evaluated also did not significantly differ from that of the frozen-thawed sperm without sex-selection. The comparable results between PureSperm® and Percoll are in agreement with the study conducted in human sperm where 4-layer gradients of Percoll and PureSperm® were compared [25]. Moreover, the unimproved quality of sperm after gradient selection was the same phenomena as found previously [14,26]. This likely resulted from that the sperm to be processed (i.e. fresh collected sperm) could be considered as the high quality sperm. Therefore, sperm selection with gradient centrifugations would have very little effects on improving sperm quality. Maxwell et al. [14] stated that the improvement in total motility after gradient separation was obtained when initial samples containing low sperm motility were included. In contrast to PureSperm® and Percoll, X-sperm selection with OptiPrep™ had an adverse effect on the percentages of motile, viable, morphologically normal acrosome, and membrane intact sperm (see in Table 1). The negative effect of iodixanol, an active ingredient of OptiPrep™, on sperm quality has been reported in human sperm [12,27].

However, the similar and even better sperm quality were also demonstrated in samples separated by iodixanol, compared to Percoll [17,28]. Differences in experimental conditions, concentrations of iodixanol used, the centrifugation time and force, and even species of sperm donors might be responsible for such controversial results. Smith et al. [28] indicated that the percentages of motile sperm and sperm with normal morphology can dramatically improve by modifying the iodixanol gradient volumes, the centrifugation force and the duration of the centrifugation.

It has been suggested that during IVP of embryos, components of the *in vitro* culture (IVC) medium have an influence on the survival of male and female embryos in different levels. For instance, glucose contained in the culture medium could inhibit the development of female embryos more than that of the male counterparts [29]; on the other hand, a high number of female embryos have been investigated when using mSOF supplemented with citrate and myo-inositol as the IVC medium [30]. The latter observation was supported by the results of our study where mSOF plus 0.3 mM Na-citrate and 2.8 mM myo-inositol were used during IVC, and sex of embryos in the control group deviated from the theoretical ratio (50 : 50) to 41.2 males : 58.8 females despite doing IVF with non-sex-sorted sperm. Therefore, it is not surprising that the sex ratio of *in vitro* produced embryos would not be approximately 50 : 50 and may differ from the ratio of male to female calves born, although the same ejaculated semen was tested [31].

After gradient centrifugation, the ratio of X-sperm in the samples selected by Percoll did not significantly alter in comparison to the non-centrifuged samples (see in Table 2). This result was not in accordance with some previous findings such as Kobayashi et al. [2], that multilayer Percoll gradients could successfully enrich X-bearing bovine sperm. In our opinion, the time (20 min) and/or force (500 X g) of centrifugation applied in the present condition did possibly exceed those needed to separate two genders of sperm in the tested gradient, i.e. Percoll. This assumption could be supported by the very high sperm recovery rate showed in the Percoll group (86.5%) which was 36.5% over the theoretical value, i.e. approximately 50% of X-sperm contained in each ejaculate. This implies that besides X-sperm desired, unwanted Y-sperm in the considerable extent must be included in the sperm pellet remaining after centrifugation. The mechanism of selection of X-bearing sperm by discontinuous gradient centrifugations is not fully understood [2]. However, it is believed that separation occurs as a result of difference in DNA content between X- (heavier) and Y- (lighter) bearing sperm, which in theory heavier sperm reach the bottom of the tube more and faster than lighter ones after centrifugation [32]. In cattle, the difference is very little (i.e. 3.8%) and is close to the minimal level (3.5%) necessary for separation to occur [33]. The time and force of centrifugation used in this species should thereby be more concerned for achieving sperm separation.

Our results showed that the sex ratio of *in vitro* produced bovine embryos in the PureSperm group was not significantly different from the control, non-sex-sorted samples. This indicated that 8-layer gradient centrifugation in an alternative gradient medium named PureSperm® (colloidal silane-coated silica particles) seemed not to be effective for separating X-bearing bovine sperm. This gradient solution, to our knowledge, has only been used to select sperm with good quality [27] but has never been applied in sex selection of sperm in any species including bovine. The possible mechanism by which PureSperm® excludes damaged and dead sperm is through either an interaction between the particles in the silica colloid and the sperm membrane proteins or the physical properties of the colloid that help agglomerate immotile cells [26]. Similar to PureSperm®, the present study found that discontinuous 8-layer OptiPrep gradients were not able to increase the X-sperm ratio in post-centrifuged samples. The present observation agreed with Resende et al. [17] who used IVP of embryos and PCR to evaluate efficacy of OptiPrep™ in sex separation of bovine sperm and could not demonstrate a change in the proportion of X-bearing sperm in the samples centrifuged through continuous OptiPrep gradients. Nevertheless, some more experiments with modifying the sperm separation protocol such as the centrifugation time and force, concentrations of gradient solution, and volume of solution per layer ought to be tried prior to draw conclusions on the PureSperm and OptiPrep' s efficacy.

In the present study, the sex of sperm both in the control and the centrifuged samples was identified indirectly in *in vitro* produced embryos by using multiplex PCR. *In vitro* embryo production and subsequently PCR however may not be the most appropriate techniques to evaluate effectiveness of the gradient media in sperm sex separation. It is owing to that using these techniques, only processed sperm with the capability of fertilizing oocytes and furthermore only fertilized oocytes with the capability to develop into embryos were evaluated their sex, while the sperm and the fertilized eggs lacking these abilities were neglected. Therefore, sperm sex determination with direct but more complicated and more expensive methods such as quantitative real-time PCR (qPCR) and multicolour fluorescence *in situ* hybridization (FISH) may additionally be conducted in order to confirm the results. Alhough PureSperm®, OptiPrep™ and Percoll were all not able to separate X- from Y-bearing sperm in the present study they did not interfere development of embryos. This could be recognized by the similarity in the cleavage rates of embryos between the control (I) and the centrifuged samples (II, III, and IV). Our finding helps support the manufacturers' claim that these density gradient media contain low endotoxin levels and are non-toxic to cells, at least in the *in vitro* bovine embryos.

In conclusions, centrifugation of fresh bovine semen in PureSperm® did not damage the survival of sperm after cryopreservation. However on the basis of testing in the *in vitro* produced embryos by multiplex PCR, 8-layer PureSperm® and OptiPrep™ density gradients were not able to deviate the sex ratio of bovine sperm.

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