รายงานวิจัยฉบับสมบูรณ์

โครงการ: การคัดแยกเพศอสุจิของโคนมด้วยวิธี discontinuous PureSperm® gradients

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กรกฎาคม 2556

สัญญาเลขที่ TRG5580005

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(ความเห็นในรายงานฉบับนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

โครงการวิจัยนี้สามารถสำเร็จลุล่วงได้ด้วยความกรุณาของบริษัท ฟาร์มโชคชัย จำกัด อำเภอปาก-ช่อง จังหวัดนครราชสีมา ที่ให้ความอนุเคราะห์ในด้านสถานที่และตัวอย่างพ่อโคที่ใช้ในงานวิจัย อีกทั้งเจ้าหน้าที่แผนกพัฒนาสายพันธุ์โคของฟาร์มฯ ทุกท่านที่ช่วยอำนวยความสะดวกในระหว่างทำวิจัยที่ฟาร์มฯ คณะผู้วิจัยขอขอบคุณบุคคลากรประจำกลุ่มวิจัยชีววิทยาระบบสืบพันธุ์ สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล ทุกท่านที่ช่วยอำนวยความสะดวกเรื่องการเดินทาง การเก็บตัวอย่างไข่แม่โคจากโรงฆ่าสัตว์ การจัดเตรียมและทำความสะอาดอุปกรณ์ที่ใช้ในงานวิจัย และขอขอบคุณทุกท่านที่มิได้กล่าวถึง ณ ที่นี้ที่มีส่วนช่วยให้โครงการวิจัยนี้สำเร็จไปด้วยดี เหนือสิ่งอื่นใดคณะผู้วิจัยขอขอบพระคุณสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) และมหาวิทยาลัยมหิดลที่ให้โอกาสแก่คณะผู้วิจัยและให้การสนับสนุนด้านงบประมาณที่ใช้ในโครงการวิจัยนี้

คณะผู้วิจัย

บทคัดย่อ

รหัสโครงการ: TRG5580005

ชื่อโครงการ:การคัดแยกเพศอสุจิของโคนมด้วยวิธี discontinuous PureSperm® gradients

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งานวิจัยชิ้นนี้มีขึ้นเพื่อศึกษาถึงผลของการปั่นเหวี่ยงน้ำเชื้อโคนมผ่านชั้นต่างระดับความเข้มข้นของสาร PureSperm® และ OptiPrep™ ที่มีต่อคุณภาพเชื้ออสุจิและสัดส่วนของเพศตัวอ่อนที่ได้ โดยแบ่งน้ำเชื้อสดที่รีดได้จากโคนมแต่ละตัว (n=12) ออกเป็น 4 กลุ่ม ได้แก่ **กลุ่มควบคุม** (I) เป็นน้ำเชื้อที่ไม่ผ่านกระบวนการคัดแยกเพศ ซึ่งจะถูกนำไปแช่แข็งกับสารละลายทริสและไข่แดง **กลุ่มคัดแยกเพศ** เป็นน้ำเชื้อที่ถูกนำไปปั่นเหวี่ยง (500g นาน 20 นาที) ผ่านชั้นต่างระดับความเข้มข้น (จำนวน 8 ชั้น) ของสาร PureSperm® (II), OptiPrep™ (III) และ Percoll (IV) ก่อนนำตะกอนที่กันหลอดซึ่งคาดว่าเป็นเชื้ออสุจิเพศเมียไปแช่แข็งด้วยสารละลายชนิดเดียวกันกับกลุ่มควบคุม นำตัวอย่างแช่แข็งทั้ง 4 กลุ่มมาอุ่นละลาย ตรวจคุณภาพและนำไปปฏิสนธิกับไข่ในห้องปฏิบัติการ ตรวจหาเพศของตัวอ่อนด้วยวิธีปฏิกิริยาลูกโซ่โพลิเมอเรส พบว่าหลังอุ่นละลาย ร้อยละของอสุจิมีชีวิต อสุจิมีรูปร่างอโครโซมเป็นปกติและอสุจิมีเยื่อหุ้มสมบูรณ์ในกลุ่ม PureSperm® (II) และ Percoll (IV) มีค่าใกล้เคียงกับกลุ่มควบคุม (I) ($P>0.05$) และมีค่ามากกว่ากลุ่ม OptiPrep™ (III) อย่างมีนัยสำคัญ ($P<0.0001$ ถึง $P=0.03$) สัดส่วนของตัวอ่อนเพศเมียที่ได้จากอสุจิซึ่งผ่านการปั่นเหวี่ยงด้วยสารทั้ง 3 ชนิดมีค่าไม่แตกต่างกับค่าของกลุ่มควบคุม (58.8%, 61.6%, 61.0% และ 54.3%, สำหรับตัวอย่างกลุ่ม I, II, III และ IV ตามลำดับ, $P>0.05$) จึงได้ข้อสรุปว่า การปั่นเหวี่ยงน้ำเชื้อโคนมผ่านชั้นต่างระดับความเข้มข้นของสาร PureSperm® นั้นไม่ส่งผลเสียต่อคุณภาพของเชื้ออสุจิ อย่างไรก็ดี สาร PureSperm® และ OptiPrep™ ไม่สามารถทำให้สัดส่วนของตัวอ่อนเพศเมียในตัวอย่างน้ำเชื้อที่ผ่านการปั่นเหวี่ยงเพิ่มสูงขึ้นกว่ากลุ่มที่ไม่ผ่านการปั่นเหวี่ยงได้

คำหลัก: โค การปั่นเหวี่ยง อสุจิเพศเมีย การปฏิสนธิในหลอดแก้ว พีซีอาร์

Abstract

Project Code: TRG5580005

Project Title: Separation of X- and Y-bearing bovine sperm through discontinuous PureSperm® gradients, evaluated by *in vitro* embryo production and polymerase chain reaction (PCR)

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Project Period: 1 year (July 2012 – July 2013)

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® (II), OptiPrep™ (III), or Percoll (IV). 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: Bovine, Centrifugation, X-bearing sperm, IVF, PCR

เนื้อหางานวิจัย

บทนำ

Artificial insemination (AI) is an insemination technique which has been most widely used for propagating cattle population especially dairy cattle (Webb 2003). 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%) (Kobayashi et al. 2004). 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 (Rath and Johnson 2008).

Methods of separating X- from Y-bearing sperm are composed of, for example, discontinuous albumin gradient centrifugation (Ericsson et al. 1973), Percoll gradient centrifugation (Iizuka et al. 1987), and flow cytometry (Johnson et al. 1993). 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 (Cran and Johnson 1996). 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 (Arora et al. 1994). 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 (Rath and Johnson 2008). 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 (Claassens et al. 1998). The advantages of PureSperm® over other sperm selection gradients such as Percoll have also been reported in isolating ram (O'Brien et al. 2003) and bovine (Maxwell et al. 2007), 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 (McCann and Chantler 2001). This substance has been proven to be safety for using in human due to its low endotoxin level (Harrison 1997). 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™ (Harrison 1997; Resende et al. 2009). 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 (Resende et al. 2009). 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).

วิธีการทดลอง

Animals: At Farm Chokchai in Nakornratchasima 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.

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.

Preparation of density gradients:

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 $CaCl_2$, 3.1 mM KCl, 0.4 mM $MgCl_2$, 100 mM NaCl, 25 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 1 mM Na-pyruvate, 21.6 mM Na-lactate, 10 mM HEPES, 6 mg/ml BSA (fraction V), and 50 µg/ml gentamycin (pH=7.4)]. 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.

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.

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.

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 and described briefly as follows.

Collected fresh semen containing the sperm motility of $\geq$ 65% was further evaluated its quality in the aspects of the sperm concentration, the sperm viability, the acrosome morphology, and the 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 x 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 500g 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 300g for 10 min. The washed sperm pellets (0.5 ml each) were further frozen.

Sperm freezing and thawing: The untreated control sample (I) and the sorted sperm (II, III and IV) were frozen with Tris-egg yolk extender. Briefly, 1 ml of the samples was extended at room temperature in 3 ml of Tris-egg yolk extender [3.0 g Tris, 1.7 g citric acid, 1.3 g fructose, 0.1 g penicillin-streptomycin, 8% (v/v) glycerol and 20% (v/v) egg yolk]. The diluted samples were gradually cooled to 4^{o}C and further kept at this temperature for 4 h. At 4^{o}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 day until thawing. The straws were thawed in water at 37^{o}C for 30 s.

Evaluation of fresh and/or frozen-thawed semen quality:

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

Sperm concentration: The concentration of each ejaculate was determined with a photometer (SpermaCue^{TM}, MinitÜb GmbH, Tiefenbach, Germany).

Sperm motility: The individual progressive motility of sperm was assessed subjectively by the same technician using a bright field microscope.

The sperm viability and acrosome morphology: These two parameters were evaluated independently under a light microscope (1000x) following eosin-nigrosin staining. In brief, 5 µl of the sample were placed on a slide with a drop of eosin-nigrosin stain and then mixed together. The stained sample was smeared on a glass slide and dried. 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.

Sperm plasma membrane integrity: The integrity of sperm plasma membrane was assessed using the hypo-osmotic swelling test (HOST). 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 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.

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* after oocyte fertilization with frozen-thawed sperm in groups I to IV. The embryos' genetic sex was identified using multiplex PCR. 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.

***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 µg/ml gentamycin (A.N.B. Laboratories Co., Ltd., Bangkok, Thailand) 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^{TM}, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), 0.2 mM Na-pyruvate, 5 µg/ml LH, 0.5 µg/ml FSH, 1 µg/ml estradiol 17ß, and 40 µg/ml gentamycin. The oocytes were matured *in vitro* by incubating at 39^{o}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 µg/ml heparin (Leo, Leo Pharmaceutical Products, Ballerup, Denmark), 10 mM caffeine 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 ($1x10^6$/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, 2.8 mM myo-inositol, 0.3 mM Na-citrate, 45 µl/ml BME amino acid 50X, 5 µl/ml MEM amino acid 100X, 5% FBS and 50 µg/ml gentamycin). 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.

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. 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), 10 µM BY (the bovine Y-specific primers), 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, then stained with ethidium bromide, and finally visualized under an UV illuminator.

The flow charts depicting the experimental design are shown in figure 1.

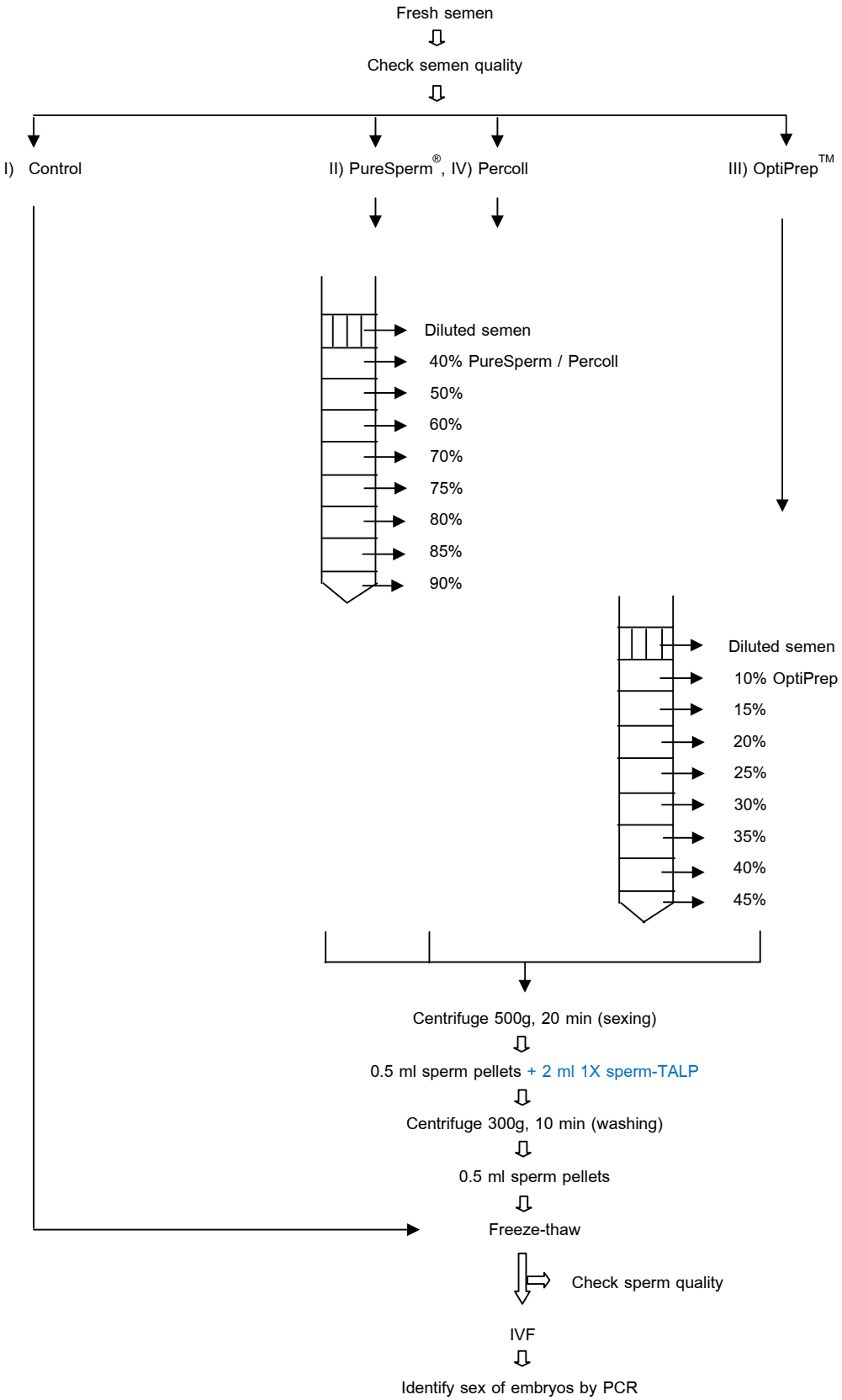

Figure 1. The experimental design demonstrating main steps conducted in this study

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 developmental 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 $P \leq 0.05$.

ผลการทดลอง

On average of twelve samples collected, the semen volume and the sperm concentration were 4.7 ± 0.4 ml and 1.9±0.1 $x10^9$ 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.

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 developmental rates of embryos to 2-4 cells, 8-16 cells, morulae and blastocysts stages observed on day 7 in the centrifuged samples were not different from the non-centrifuged sample ($P>0.05$). The data are presented in Table 2. 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 3, and an example of PCR analysis results on embryonic DNA samples observed in the agarose gel is depicted in figure 2.

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]sperm with plasma membrane intact. Value (mean±SEM) with different superscripts (^{a,b,c}) indicate significant difference within rows ($P \leq 0.05$)

Table 2. Development of in vitro-produced bovine embryos using non-sex-sorted and sex-sorted sperm of 12 samples

Groups	No. of fertilized	No. of embryos (% ± SEM)			
	oocytes	2-4 cells	8-16 cells	morulae	blastocysts
I) Control	492	404 (79.5±3.9)	333 (66.6±4.3)	192 (39.2±3.8)	60 (12.5±3.5)
II) PureSperm	497	399 (80.0±4.1)	338 (67.3±4.4)	193 (38.8±3.7)	33 (7.2±2.1)
III) OptiPrep	489	386 (78.4±4.1)	311 (63.3±4.4)	157 (32.5±4.4)	43 (8.8±4.1)
IV) Percoll	477	381 (79.9±3.8)	313 (66.3±4.7)	177 (36.5±3.6)	44 (8.1±2.0)

There were no significant differences in the values among rows within each column

Table 3. 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 No. of embryos	No. 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.

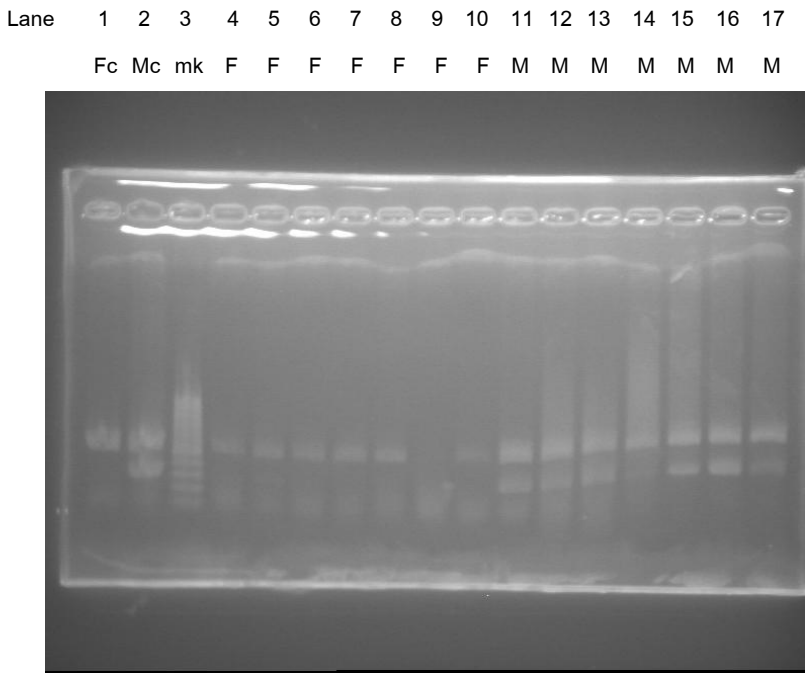

Figure 2. An example of PCR analysis results on embryonic DNA samples (Lane 4 – 17). Lanes 1 and 2 are positive controls made with female (Fc) and male (Mc) bovine genomic DNA as template. Lane 3 represents DNA size marker (mk). Lanes 4 – 10 are female embryos (F). Lanes 11 – 17 are male embryos (M).

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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 (Soderlund and Lundin 2000). Moreover, the unimproved quality of sperm after gradient selection was the same phenomena as found previously (Januskauskas et al. 2005; Maxwell et al. 2007). 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. (2007) 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 (Claassens et al. 1998; Mousset-Simeon et al. 2004). However, the similar and even better sperm quality was also demonstrated in samples separated by iodixanol, compared to Percoll (Resende et al. 2009; Smith et al. 1997). 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. (1997) 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 (Gutierrez-Adan et al. 2001); 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 (Sattar et al. 2011). 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 (Massip et al. 1996).

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 3). This result was not in accordance with some previous findings such as Kobayashi et al. (2004), that multilayer Percoll gradients could successfully enrich X-bearing bovine sperm. In our opinion, the time (20 min) and/or force (500g) 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 selection of X-bearing sperm by discontinuous gradient centrifugations is believed to occur 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 (Wolf et al. 2008). In cattle, the difference is very little (i.e. 3.8%) and is close to the minimal level (3.5%) necessary for separation (Johnson 2000). 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 with this experimental condition, 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 (Mousset-Simeon et al. 2004) 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 (Januskauskas et al. 2005). 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. (2009) 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; Resende et al. 2011) and multicolor fluorescence in situ hybridization (FISH; Habermann et al. 2005) 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 developmental 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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ภาคผนวก

SEPARATION OF X- AND Y- SPERM IN DAIRY CATTLE THROUGH DISCONTINUOUS PURESPERM® AND OPTIPREP™ GRADIENT CENTRIFUGATIONS

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Abstract: The efficacy of PureSperm® and OptiPrep™ in separation of X-bearing bovine sperm was evaluated in the *in vitro* produced embryos by multiplex polymerase chain reaction (PCR). 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® (II), OptiPrep™ (III), or Percoll (IV). 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 indirectly in the embryos. 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, PureSperm® and OptiPrep™ gradient centrifugations were not able to deviate the sex ratio of bovine sperm.

Keywords: bovine, centrifugation, X-bearing sperm, IVF, PCR

Effect of discontinuous PureSperm® and OptiPrep™ gradient centrifugations on the quality of bovine sperm and the sex ratio of *in vitro* produced embryos

Abstract

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® (II), OptiPrep™ (III), or Percoll (IV). 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: Bovine; Centrifugation; X-bearing sperm; IVF; PCR

1. Introduction

Artificial insemination (AI) is an insemination technique which has been most widely used for propagating cattle population especially dairy cattle (Webb 2003). 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%) (Kobayashi et al. 2004). 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 (Rath and Johnson 2008).

Methods of separating X- from Y-bearing sperm are composed of, for example, discontinuous albumin gradient centrifugation (Ericsson et al. 1973), Sephadex gel filtration (Steeno et al. 1975), Percoll gradient centrifugation (Iizuka et al. 1987), swim-up procedures (Check et al. 1989), and flow cytometry (Johnson et al. 1993). 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 (Cran and Johnson 1996). 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 (Arora et al. 1994; Wang et al. 1994). 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 (Rath and Johnson 2008). 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 (Claassens et al. 1998). The advantages of PureSperm® over other sperm selection gradients such as Percoll have also been reported in isolating ram (O'Brien et al. 2003) and bovine (Maxwell et al. 2007), 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 (McCann and Chantler 2001). This substance has been proven to be safety for using in human due to its low endotoxin level (Harrison 1997). 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™ (Harrison 1997; Resende et al. 2009). 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 (Resende et al. 2009). 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. Materials and methods

All media components used in this study were obtained from Sigma-Aldrich (St Louis, MO, USA) unless otherwise indicated.

2.1. Animals

At Farm Chokchai in Nakornratchasima 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 $CaCl_2$, 3.1 mM KCl, 0.4 mM $MgCl_2$, 100 mM NaCl, 25 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 1 mM Na-pyruvate, 21.6 mM Na-lactate, 10 mM HEPES, 6 mg/mL BSA

(fraction V), and 50 µg/mL gentamycin (pH=7.4, Parrish et al., 1986). 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 (Iizuka et al. 1987) and described briefly as follows.

Collected fresh semen containing the sperm motility of $\geq$ 65% was further evaluated its quality in the aspects of the sperm concentration, the sperm viability, the acrosome

morphology and the 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 x 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. (2011). Briefly, the samples were extended at room temperature in Tris-egg yolk extender [3.0 g Tris, 1.7 g citric acid, 1.3 g fructose, 0.1 g penicillin-streptomycin, 8% (v/v) glycerol and 20% (v/v) egg yolk] to obtain a final concentration of 120 x 10^6 sperm/mL. The diluted samples were gradually cooled to 4^{o}C and further kept at this temperature for 4 h. At 4^{o}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 day until thawing. The straws were thawed in water at 37^{o}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^{TM}, 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.

2.6.4. The sperm viability and acrosome morphology

These two parameters were evaluated independently under a light microscope (1000x) following eosin-nigrosin staining (Dott and Foster 1972). 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.

2.6.5. Sperm plasma membrane integrity

The integrity of sperm plasma membrane was assessed using the hypo-osmotic swelling test (HOST) (Perez-Llano et al. 2001). 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 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.

2.6.6. 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. (2005), after oocyte fertilization with frozen-thawed sperm in groups I to IV. The embryos' genetic sex was identified using multiplex PCR (Rattanasuk et al. 2011). 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.

2.7. 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 µg/mL gentamycin (A.N.B. Laboratories Co., Ltd., Bangkok, Thailand) 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^{TM}, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), 0.2 mM Na-pyruvate, 5 µg/mL LH, 0.5 µg/mL FSH, 1 µg/mL estradiol 17ß, and 40 µg/mL gentamycin. The oocytes were matured *in vitro* by incubating at 39^{o}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 µg/mL heparin (Leo, Leo Pharmaceutical Products, Ballerup, Denmark), 10 mM caffeine 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 (1x10^{6}/ml of medium); the co-culture (sperm+oocytes) was incubated at 39^{o}C in 5% CO_2 in air and saturated humidity for approximately 20 h. Following IVF, presumptive 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, 2.8 mM myo-inositol, 0.3 mM Na-citrate, 45 µL/mL BME amino acid 50X, 5 µL/mL MEM amino acid 100X, 5% FBS and 50 µg/mL gentamycin; modified from

Holm et al., 1999). Zygotes were incubated in Petri dishes under mineral oil in 5% CO_2 in air at 39^{o}C and saturated humidity for 7 d. The developed embryos (≥2-cell-stage) were determined their sex by multiplex PCR (Rattanasuk et al. 2011).

2.8. Multiplex polymerase chain reaction

Single bovine embryos placed in 6 µL DNAse/RNAse-free distilled water were extracted DNA through lysing at 95^{o}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. (2011) 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), 10 µM BY (the bovine Y-specific primers), 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^{o}C for 2 min, 45 cycles of 95^{o}C for 20 s, annealing at 52^{o}C for 45 s and extension at 72^{o}C for 50 s followed by final extension at 72^{o}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, then stained with ethidium bromide, and finally visualized under an UV illuminator.

2.9. Statistical analysis

Statistical Package for the Social Sciences (SPSS Statistics 17.0, Chicaco, IL) was used for statistical analyses. 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 $x10^9$ 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.

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.

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 (Soderlund and Lundin 2000). Moreover, the unimproved quality of sperm after gradient selection was the same phenomena as found previously (Januskauskas et al. 2005; Maxwell et al. 2007). 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. (2007) 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 (Claassens et al. 1998; Mousset-Simeon et al. 2004). However, the similar and even better sperm quality was also demonstrated in samples separated by iodixanol, compared to Percoll (Resende et al. 2009; Smith et al. 1997). 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. (1997) 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 (Gutierrez-Adan et al. 2001); 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 (Sattar et al. 2011). 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 (Massip et al. 1996).

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. (2004), 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 selection of X-bearing sperm by discontinuous gradient centrifugations is believed to occur 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 (Wolf et al. 2008). In cattle, the difference is very little (i.e. 3.8%) and is close to the minimal level

(3.5%) necessary for separation (Johnson 2000). 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 with this experimental condition, 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 (Mousset-Simeon et al. 2004) 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 (Januskauskas et al. 2005). 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. (2009) 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; Resende et al. 2011) and multicolor fluorescence in situ hybridization (FISH; Habermann et al. 2005) 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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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]sperm with plasma membrane intact. Value (mean±SEM) with different superscripts (^{a,b,c}) indicate significant difference within rows ($P \leq 0.05$)

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.

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