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Centrifugation on Percoll density gradient enhances motility, membrane integrity and *in vitro* fertilizing ability of frozen–thawed boar sperm

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Summary

The effects of Percoll density gradient centrifugation on sperm quality, *in vitro* fertilizability and developmental capacity of frozen–thawed boar sperm were evaluated. Two-step density gradient centrifugation by Percoll enhanced significantly the motility parameters of sperm compared with a simple centrifugation procedure. Percentages of motile sperm and sperm with intact plasma and acrosome membranes after Percoll separation were significantly greater than those after simple centrifugation. The rates of penetration, cleavage and blastocyst formation after *in vitro* fertilization were significantly improved by Percoll separation compared with simple centrifugation and were influenced positively by the intactness of sperm head membranes, but not any sperm motility parameters. However, insemination with increased concentrations of sperm prepared by Percoll gradient centrifugation did not improve the success of fertilization and embryo development *in vitro*. Our results indicate that the integrity of sperm head membranes after Percoll separation is important for successful embryo development *in vitro*, more so than sperm motility.

Keywords: Acrosomal membrane, *In vitro* fertilization, Plasma membrane, Sperm motility

Introduction

In vitro fertilization (IVF) systems using frozen–thawed sperm are effective for repeatable research on embryo development and fertility testing prior to commercial use of frozen semen. Sperm quality indicated by motility, viability, morphology and acrosome status is commonly thought essential for the success of IVF and subsequent embryo development in humans (Donnelly *et al.*, 1998; Salumets *et al.*, 2002), cattle (Brito *et al.*, 2003; Cesari *et al.*, 2006) and pigs (Gadea, 2005; Gil *et al.*, 2005, 2008). However, cryopreservation of boar sperm influences motility (Rath & Niemann, 1997; Peña *et al.*, 2003; Flores *et al.*, 2008) and membrane condition (Bailey *et al.*, 2000; Curry, 2000; Peña *et al.*, 2003; Flores *et al.*, 2008) due to damage during freezing and thawing. Thus, the separation of sperm with superior quality from frozen–thawed semen is an important step to improve the efficiency of IVF.

Percoll® (a colloidal suspension of polyvinyl pyrrolidone-coated silica particles) density gradients are widely used for the selection of sperm with superior quality by centrifugation from man (Drobnis *et al.*, 1991), bull (Suzuki *et al.*, 2003; Oliveira *et al.*, 2011), boar (Grant *et al.*, 1994; Suzuki & Nagai, 2003; Martecikova *et al.*, 2010; Matás *et al.*, 2011) and ram (Valcárcel *et al.*, 1996) semen. In the pig, sperm with high motility (Grant *et al.*, 1994; Martecikova

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et al., 2010; Matás *et al.*, 2011) and intact plasma and acrosomal membranes (Martecikova *et al.*, 2010) were separated by Percoll gradient centrifugation from fresh or frozen–thawed semen. Furthermore, rates of penetration (Matás *et al.*, 2011), cleavage (Grant *et al.*, 1994; Jeong & Yang, 2001) and blastocyst formation (Jeong & Yang, 2001) after IVF and embryo culture were improved by insemination with boar sperm prepared by Percoll gradient centrifugation. However, it is still unclear that the relationship between sperm parameters after Percoll centrifugation and *in vitro* fertilizability and developmental capacity of frozen–thawed boar spermatozoa.

The wide variation in sperm parameters among boars or between ejaculates of individual boars appears to influence the incidence of penetration and polyspermy *in vitro* (Xu *et al.*, 1996a,b; Suzuki *et al.*, 2005). While Percoll separation of cryopreserved epididymal (Suzuki & Nagai, 2003; Suzuki *et al.*, 2005) and ejaculated (Suzuki *et al.*, 2005) sperm has widespread use in porcine IVF using various batches of semen from different boars, differences in success of fertilization (Suzuki & Nagai, 2003; Suzuki *et al.*, 2005) and embryo development *in vitro* (Suzuki *et al.*, 2005) among the batches have still been observed. Salumets *et al.* (2002) reported correlations between sperm characteristics, such as sperm morphology after Percoll gradient centrifugation and the cleavage rate of embryos after IVF in humans, but it is uncertain which factor of Percoll-separated sperm influences IVF and subsequent embryo development outcomes in pigs.

The objectives were: (1) to evaluate the relationship between motility and membrane status of frozen–thawed boar spermatozoa separated by Percoll density gradient centrifugation using computer-assisted sperm analyzer (CASA) and flow cytometry; (2) to determine the effects of Percoll separation for cryopreserved semen on IVF outcomes and subsequent embryo development; and (3) to investigate whether alternative concentrations of inseminated sperm used during IVF after Percoll separation, from batches of semen having different characteristics, improves IVF and early embryonic development.

Materials and methods

Preparation of frozen–thawed boar sperm

The method of semen cryopreservation was essentially the same as that described by Yoshioka *et al.* (2003). The semen samples were collected from six boars (Duroc; $n = 3$, Large White; $n = 2$, Landrace; $n = 1$), packed in 0.5-ml plastic straws at 1×10^9 sperm/ml and stored in liquid nitrogen until use. To thaw frozen sperm, the straw was immersed in water at 38°C for 30 s.

Percoll® (GE Healthcare UK Ltd., Buckinghamshire, UK) was mixed with five-strength modified Modena solution (mMS; Sone *et al.*, 1992) supplemented with 50 μg/ml gentamicin to prepare 80% and 50% Percoll solution. The 0.5 ml of frozen–thawed semen mixed with 2 ml of 50% Percoll layered on 2 ml of 80% Percoll in a 15-ml conical tube (Sumilon, Tokyo, Japan) and was centrifuged at 700 *g* for 20 min in the Percoll group. In the control group, 0.5 ml frozen–thawed semen was mixed with 2 ml of mMS in a 15-ml conical tube and centrifuged at 500 *g* for 5 min. After centrifugation in both groups, the supernatant above the sperm fraction was carefully removed and washed twice with porcine fertilization medium (PFM; Research Institute for the Functional Peptides, Yamagata, Japan) by centrifugation at 500 *g* for 5 min. The sperm pellet at the bottom of the tube was resuspended in PFM. All sperm washing procedures were conducted in air at 38°C.

Assessment of sperm motility

The sperm motility of each sample was assessed by CASA (CEROS Sperm Analyzer, Hamilton Thorne Biosciences, Beverly, MA, USA) at 60 min after thawing. Each sample was diluted to 2×10^7 sperm/ml in PFM, and approximately 2 μl of the sperm suspension was transferred to a pre-warmed 2X-CEL disposable semen analysis slide (Hamilton Thorne) with a chamber depth of 20 μm placed on a warmed microscope stage at 37°C. At least 200 motile sperm were assessed. The different motility parameters evaluated were percentage total motility (MOT, %), percentage progressive motility (PROG, %), average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), straightness (STR = VSL/VAP, %) and linearity (LIN = VSL/VCL, %).

Assessment of plasma and acrosome membrane integrity

The integrity of the plasma and acrosomal membranes was evaluated at 60 min after thawing following the procedure described by Mercado *et al.* (2009) with some modifications. Each sample after washing was diluted to 1×10^7 sperm/ml in PFM. Next, 25 nM of SYBR-14 (Component A of LIVE/DEAD Sperm Viability Kit, Molecular Probes, Eugene, OR, USA), 12 μM of propidium iodide (PI) (Component B of LIVE/DEAD sperm Viability Kit) and 0.25 mg/ml of Alexa Fluor 647-conjugated peanut agglutinin (PNA) (Molecular Probes) at final concentrations were simultaneously added to diluted semen. Samples were mixed and incubated at 38°C for 10 min. Stained sperm cells subsequently were analyzed in a flow

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cytometer (FACSAria™, Becton Dickinson, Franklin Lakes, NJ, USA). In total, 50,000 events with forward and side scatter properties of sperm cells were gated initially. Then, dots that were double-negative for SYBR-14 and PI were further gated out as non-sperm particles. Sperm cells were divided into three subpopulations: sperm with intact plasma and intact acrosomal membrane (IPIA; PI (−) and PNA (−)); sperm with a damaged plasma membrane (DP; PI (+)); and sperm with a damaged acrosomal membrane (DA; PNA (+)).

In vitro maturation (IVM), IVF and *in vitro* culture (IVC)

The *in vitro* production (IVP) of porcine blastocysts was essentially the same as that previously described by Mito *et al.* (2009) with some modifications. Cumulus–oocyte complexes (COCs) were aspirated from antral follicles (3–6 mm in diameter) of ovaries from slaughtered prepubertal gilts. After washing the COCs twice in porcine oocyte/embryo collection medium (POE-CM; Research Institute for the Functional Peptides), only COCs with uniform ooplasm and a compact cumulus cell mass were selected for IVM. The COCs were matured in porcine oocyte medium (Research Institute for Functional Peptides) supplemented with 10 ng/ml recombinant human transforming growth factor-α (R&D Systems Inc., Minneapolis, MN, USA), 0.5 IU/ml recombinant human follicle-stimulating hormone (FSH) (Gonalef®, Merck Serono SA, Aubunne, Switzerland) and 1 mM dibutyryl cyclic AMP (Sigma Chemical Co., St. Louis, MO, USA) for 20 h, and subsequently matured in the same medium without FSH and dibutyryl cyclic AMP for a further 24 h.

Matured COCs at 44 h after IVM were co-incubated with centrifuged- or Percoll-separated-sperm at a concentration of 1×10^6 cells/ml in 100-μl droplets of PFM for 10 h. The COCs were also inseminated with sperm at a concentration of 2×10^6 cells/ml in an experiment to determine the effect of concentrations of spermatozoa prepared by Percoll gradient centrifugation on IVF and IVC outcomes. Each droplet contained 15–20 COCs.

After IVF, the presumptive zygotes were stripped of cumulus cells by vortexing for 4 min in POE-CM. Presumptive zygotes were washed twice with POE-CM and porcine zygote medium-5 (PZM-5; Research Institute for the Functional Peptides) and cultured in 50-μl droplets of PZM for 100−105 h. Each droplet contained 25–30 presumptive zygotes. IVM, IVF and IVC were maintained at 39°C in a humidified atmosphere containing 5% CO_2: 5% O_2: 90% N_2.

Evaluation of fertilization and embryo development

Denuded presumptive zygotes after vortexing were mounted on glass slides, fixed for 24–48 h in 25% (v/v) acetic acid in ethanol, stained with 1% (w/v) orcein in 45% (v/v) acetic acid, and examined under a phase-contrast microscope (Yoshioka *et al.*, 2008). Oocytes were considered as fertilized when they had one or more swollen sperm head(s) and/or male pronuclei with corresponding sperm tails. The frequency of normal fertilization was determined as the ratio of oocytes with a second polar body, a pair of pronuclei and corresponding sperm tail by the total number of oocytes evaluated. The rates of cleavage (≥ 2-cell stage) and blastocyst formation were evaluated under a stereomicroscope on days 2 and 5 (day 0 = the day of IVF), respectively. The total cell number of blastocysts was assessed on day 5 using an air-drying method that has been described in detail elsewhere (Yoshioka *et al.*, 2003).

Statistical analysis

Data of sperm characteristics, IVF and IVC were analyzed using the SAS general linear model (GLM) procedure (SAS Institute Inc., Cary, NC, USA). The statistical model included the effect of boar, treatment and boar × treatment interaction. When a significant effect was detected by analysis of variance, the significance of difference between means was determined by Tukey's test. A value of $P < 0.05$ was considered to be significant.

The influence of motility parameters and the plasma and acrosome membrane statuses in Percoll-separated spermatozoa on IVF and IVC outcomes was analyzed with the SAS MIXED procedure. IVF and IVC data were analyzed according to statistical model, including the fixed effect of sperm motility parameters (MOT, PROG, VAP, VSL, ALH, STR, BCF and LIN) and the plasma membrane and acrosomal status of sperm (IPIA, DP and DA), and the random effect of boar. A P-value < 0.05 was considered to be significant.

Results

Effects of Percoll separation on sperm motility

Parameters of sperm motility assessed by CASA are presented in Table 1. MOT and PROG of Percoll-separated sperm were higher than those of washed sperm ($P < 0.05$). VAP, VSL and VCL in the Percoll group were faster than those in the control group ($P < 0.05$). STR and LIN in the Percoll group were also higher than those in the control group ($P < 0.05$). BCF in the Percoll group was lower than that in the control

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Table 1 Effects of Percoll separation on spermatozoa motility

Parameters	Control	Percoll separation
Total number of counted sperm	225.5 ± 5.6	228.1 ± 5.3
Motile (%)	55.2 ± 3.9[a]	92.1 ± 2.0[b]
Progressive (%)	12.5 ± 1.4[a]	51.8 ± 2.7[b]
Average path velocity (VAP) (μm/s)	42.0 ± 1.4[a]	71.1 ± 2.2[b]
Straight line velocity (VSL) (μm/s)	31.1 ± 1.6[a]	62.5 ± 2.1[b]
Curvilinear velocity (VCL) (μm/s)	74.7 ± 1.7[a]	96.3 ± 2.6[b]
Amplitude of lateral head displacement (ALH) (μm)	3.7 ± 0.2	3.7 ± 0.1
Beat cross frequency (BCF) (Hz)	36.0 ± 0.5[a]	31.5 ± 0.5[b]
Straightness (STR) (%)	66.4 ± 1.6[a]	83.0 ± 0.6[b]
Linearity (LIN) (%)	41.9 ± 1.7[a]	62.9 ± 0.9[b]

Data from 18 replicates (three times for each of the six boars).
Values are expressed as mean ± standard error of the mean (SEM).
[a,b]Within each row, values with different superscripts are significantly different ($P < 0.05$).

Table 2 Effects of Percoll separation on plasma and acrosome integrity of sperm

Plasma and acrosomal membrane status (%)	Control	Percoll separation
Intact plasma and acrosomal membranes (IPIA)	37.2 ± 2.9[a]	65.9 ± 1.5[b]
Damaged plasma membrane (DP)	61.2 ± 2.9[a]	32.7 ± 1.4[b]
Damaged acrosomal membrane (DA)	26.7 ± 2.3[a]	10.5 ± 1.1[b]

Data from 18 replicates (three times for each of six boars).
Percentages are expressed as mean ± standard error of the mean (SEM).
[a,b]Within each row, values with different superscripts are significantly different ($P < 0.05$).

group ($P < 0.05$). ALH did not differ significantly between groups.

Effects of Percoll separation on plasma and acrosomal membrane integrity of sperm

Data on integrity of plasma and acrosomal membranes assessed by flow cytometry are shown in Table 2. A higher percentage of IPIA and lower percentages of DP and DA were observed in the Percoll group compared with the control group ($P < 0.05$).

Effects of Percoll separation on *in vitro* fertilizability and developmental capacity of sperm

Effects of Percoll separation on IVF and IVC are shown in Tables 3 and 4. The rates of sperm penetration, normal fertilization and polyspermic fertilization of oocytes in the Percoll group were greater than those in the control group ($P < 0.05$), respectively. The percentages of presumptive zygotes that cleaved and developed to the blastocyst stage were also higher in the Percoll group than those in the control group ($P < 0.05$). There was no difference in the total number of cells in blastocysts between groups.

Influences of sperm characteristics on IVF and IVC outcomes after Percoll separation

All of motility parameters of Percoll-separated sperm did not significantly affect both IVF and IVC outcomes (Table 5). The percentages of IPIA affected the rates of sperm penetration, cleavage and blastocyst formation ($P < 0.05$), respectively, while there was no significant influence of them on the rates of normal fertilization

Table 3 Effects of Percoll separation on fertilization status of porcine oocytes

Treatment	Number of oocytes examined	Number (%) of oocytes		
		Penetrated	Normal fertilized	Polyspermy
Control	719	216 (30.3 ± 3.2)[a]	139 (19.5 ± 2.1)[a]	7 (1.1 ± 0.6)[a]
Percoll separation	713	388 (54.6 ± 3.2)[b]	270 (38.1 ± 2.7)[b]	24 (3.4 ± 0.7)[b]

Data from 42 replicates (seven times for each of six boars).
Percentages are expressed as mean ± standard error of the mean (SEM).
[a,b]Within each column, values with different superscripts are significantly different ($P < 0.05$).

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Table 4 Effects of Percoll separation on early development of porcine embryos

Treatment	Number of presumptive zygotes cultured	Cleaved at day 2 (%)	Blastocysts at day 5 (%)	Total cell number in blastocysts
Control	941	240 (25.2 ± 2.6)[a]	119 (12.5 ± 1.8)[a]	47.5 ± 1.6
Percoll separation	942	453 (47.7 ± 3.0)[b]	237 (24.8 ± 2.7)[b]	47.9 ± 1.3

Data from 42 replicates (seven times for each of six boars).
Values are expressed as mean ± standard error of the mean (SEM).
[a,b]Within each column, values with different superscripts are significantly different ($P < 0.05$).

Table 5 Influences of parameters of sperm prepared with Percoll on *in vitro* fertilization (IVF) and *in vitro* culture (IVC) outcomes

Parameters	Penetration	Normal fertilization	Polyspermy	Cleavage	Blastocysts
Motile	NS	NS	NS	NS	NS
Progressive	NS	NS	NS	NS	NS
Average path velocity (VAP)	NS	NS	NS	NS	NS
Straight line velocity (VSL)	NS	NS	NS	NS	NS
Curvilinear velocity (VCL)	NS	NS	NS	NS	NS
Amplitude of lateral head displacement (ALH)	NS	NS	NS	NS	NS
Beat cross frequency (BCF)	NS	NS	NS	NS	NS
Straightness (STR)	NS	NS	NS	NS	NS
Linearity (LIN)	NS	NS	NS	NS	NS
Intact plasma and acrosomal membranes (IPIA)	$P = 0.04$	NS	NS	$P = 0.01$	$P = 0.04$
Damaged plasma membrane (DP)	$P = 0.03$	NS	NS	$P = 0.01$	$P = 0.04$
Damaged acrosomal membrane (DA)	$P = 0.05$	NS	NS	$P = 0.01$	$P = 0.04$

NS: not significant.

Table 6 Effects of concentrations of sperma prepared by Percoll gradient centrifugation on fertilization status of porcine oocytes

Boar	Sperm concentration (sperm/ml)	Number of oocytes examined	Number (%) of oocytes		
			Penetrated	Normal	Polyspermy
A	1×10^6	137	93 (67.9 ± 8.6)	53 (38.6 ± 6.5)	15 (10.9 ± 1.7)
	2×10^6	142	84 (59.2 ± 4.3)	50 (35.3 ± 4.2)	10 (7.1 ± 2.1)
B	1×10^6	142	60 (42.7 ± 5.8)	27 (19.2 ± 5.8)	8 (5.8 ± 2.8)
	2×10^6	139	48 (34.8 ± 4.9)	26 (18.9 ± 5.0)	10 (7.2 ± 1.9)
Boar			$P < 0.01$	$P < 0.01$	NS
Sperm concentration			NS	NS	NS
Boar × sperm concentration			NS	NS	NS

Data from seven replicates of each treatment.
Percentages are expressed as mean ± standard error of the mean (SEM).
NS: not significant.

and polyspermy. The proportions of DP and DA significantly affect results of IVC.

Effects of sperm concentrations after Percoll separation on IVF outcomes and early embryo development

Effects of concentrations of Percoll-separated sperm on IVF and IVC are shown in Tables 6 and 7. In this experiment, we used two batch of semen with high (69.3% ± 2.1%, Boar A) and low (56.6% ± 3.3%, Boar B) proportions of IPIA after Percoll gradient centrifugation. Sperm concentrations did not influence both IVF and IVC outcomes. However, the rates of sperm penetration, normal fertilization, cleavage and blastocyst formation differed between boars ($P < 0.05$). There was no difference between boars in the percentage of polyspermic oocytes.

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Table 7 Effects of concentrations of sperm prepared by Percoll gradient centrifugation on early development of porcine embryos

Boar	Sperm concentration (sperm/ml)	Number of presumptive zygotes cultured	Cleaved at day 2 (%)	Blastocysts at day 5 (%)
A	1×10^6	214	119 (64.2 ± 3.4)	82 (44.3 ± 4.4)
	2×10^6	218	218 (62.7 ± 5.5)	73 (38.7 ± 7.4)
B	1×10^6	214	60 (32.1 ± 4.1)	40 (21.5 ± 2.9)
	2×10^6	224	49 (25.0 ± 4.3)	25 (12.8 ± 3.0)
	Boar		$P < 0.01$	$P < 0.01$
	Sperm concentration		NS	NS
	Boar × sperm concentration		NS	NS

Data from eight replicates of each treatment.
Percentages are expressed as mean ± standard error of the mean (SEM).
NS: not significant.

Discussion

We have demonstrated that the Percoll centrifugation procedure was effective for selecting sperm with high motility, IPIA membranes from boar frozen–thawed semen. Furthermore, the use of sperm prepared by Percoll gradient centrifugation resulted in high rates of fertilization, cleavage and blastocyst production *in vitro*. However, an increase in a concentration of sperm that had a relatively low proportion of intact plasma and acrosomal membranes for IVF after Percoll centrifugation did not improve the fertilization and subsequent early embryo development *in vitro*.

Integrity of the sperm membranes is compromised markedly after freezing and thawing. It has been shown that the sperm plasma membrane after cryopreservation is injured and capacitation and/or acrosome reaction is enhanced due to elevated intracellular calcium levels of frozen–thawed sperm (Maxwell & Johnson, 1997; Bailey *et al.*, 2000). Here, the Percoll separation method enhanced recovery rate of sperm with intact plasma and acrosome membranes and high motility from frozen–thawed boar semen, as reported previously (Martecikova *et al.*, 2010). However, it has also been reported that there was no significant difference in the percentage of sperm with acrosomes that reacted in humans (Drobnis *et al.*, 1991), but was loss of acrosomes in bulls (Suzuki *et al.*, 2003) and boars (Grant *et al.*, 1994) between Percoll-separated and centrifuged semen. The duration and force of centrifugation for washing frozen–thawed boar semen influenced sperm motility and the percentage of sperm with intact acrosomes (Carvajal *et al.*, 2004). The Percoll centrifugation conditions (700 $g \times 20$ min versus 5000 $g \times 5$ min) did not affect integrity of frozen–thawed sperm membranes in bulls (Machado *et al.*, 2009). The cell suspension in the previous washing control groups were centrifuged once at 600 $g \times 5$ min (Drobnis *et al.*, 1991), once at 750 $g \times 15$ min (Suzuki *et al.*, 2003), and twice at 250 $g \times 4$ min (Grant *et al.*, 1994), respectively. Here, sperm for the control group were washed three times at 500 $g \times 5$ min. This intensive centrifugation in our study compared with the previous reports may lead to the significant difference between the Percoll and control groups, resulting in a low proportion of sperm with intact acrosomes in the control group. Thus, our results confirm that the Percoll centrifugation is capable of reducing the population of sperm with damaged membranes after cryopreservation and thawing in boar semen.

Intact sperm head membranes are a prerequisite for capacitation, subsequent hyperactivation, acrosome reaction and fertilization (Gadella *et al.*, 2008). We found that the integrity of both the plasma and acrosome membranes after Percoll separation, but not sperm motility, affected positively the rates of penetration, normal fertilization, cleavage and blastocyst formation. Even without Percoll separation of sperm, integrity of both plasma and acrosome membranes of frozen–thawed ejaculated bull sperm (Tartaglione & Ritta, 2004) or acrosome integrity of cryopreserved epididymal boar sperm (Ikeda *et al.*, 2002) rather than sperm motility had an influence on sperm penetration rate after IVF. These results suggest that sperm membrane integrity may be a valuable parameter for evaluation of fertilizability and early embryo developmental capacity *in vitro*.

Interestingly, the increase in the number of IPIA sperm per oocyte by doubling the sperm concentration during IVF did not influence the fertilization parameters and early embryo development. In general, an increase in sperm concentrations or sperm:oocyte ratio during IVF affects fertilization and subsequent early embryo development in cattle (Ward *et al.*,

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2002, 2003) and pigs (Wang *et al.*, 1991; Popwell & Flowers, 2004; Gil *et al.*, 2007). High sperm:oocyte ratio (1000−2000) improved penetration rate, resulting in low monospermy rate and high polyspermy rate compared with low sperm:oocyte ratio (500) (Gil *et al.*, 2007). In cattle, rates of fertilization, cleavage and blastocyst formation were reduced by decreasing the sperm:oocyte ratio from 10,000 to 100 (Ward *et al.*, 2002, 2003). Conversely, there was no significant change in rates of penetration and monospermic penetration (Wang *et al.*, 1991; Gil *et al.*, 2007), and percentages of cleavage and blastocyst development (Ward *et al.*, 2002, 2003) in the high sperm:oocyte ratio. Sperm concentration of between 1×10^6 and 2×10^6 /ml, or sperm:oocyte ratio of between 5000 and 10,000, may be within the range that does not affect IVF and IVC outcomes in our IVP system.

Our results suggest that the Percoll procedure enhanced the rate of penetration, normal fertilization and polyspermic fertilization of oocyte. This finding corresponds with a previous report by Matás *et al.* (2011). While polyspermic penetration remains a major obstacle in the production of normal porcine embryo *in vitro* (Funahashi, 2003), the rates of cleavage and blastocyst formation in the Percoll-separated group were enhanced compared with those in the washing group in our study and in previous reports (Grant *et al.*, 1994; Jeong & Yang, 2001). Thus, Percoll centrifugation seems to involve not only the polyspermic rate but also the proportion of normally fertilized oocytes. The increase in normal fertilization rates using frozen–thawed spermatozoa after Percoll separation for IVF may influence IVC outcomes.

The Percoll centrifugation procedure for isolation of 'good condition' sperm from frozen–thawed boar semen improves fertilization and subsequent embryo development *in vitro*. The membrane integrity of Percoll-separated frozen–thawed sperm is important for sperm penetration and embryo developmental capacity, more so than sperm motility and sperm concentration at insemination *in vitro*.

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