

## Effects of a Short Co-Incubation Time for Spermatozoa-Oocytes Using Commercial Liquid Stored Boar Semen on Porcine *in vitro* Fertilization Efficiency

Seong-Sung Kwak and Sang-Hwan Hyun

Laboratory of Veterinary Embryology and Biotechnology, College of Veterinary Medicine,  
Chungbuk National University, 361-763 Cheongju, Republic of Korea

**Abstract:** This study was conducted to evaluate if the efficiency of porcine *In Vitro* Fertilization (IVF) could be improved by a short co-incubation time for spermatozoa-oocytes using commercial Liquid Stored (LS) boar semen. For this study, LS semen was supplied weekly from the Veterinary Service Laboratory (Department of Livestock Research, Yong-in, Gyeonggi-do, Republic of Korea) and kept at 17°C for 5 days. A total of 808 immature cumulus-oocyte complexes from six replicates were matured *in vitro* and inseminated with LS semen ( $5 \times 10^5$  mL<sup>-1</sup> sperm concentration) for 5, 20, 90 or 360 min (control group). To evaluate the developmental competence of each group, all IVF embryos were examined for the following: fertilization rate, cleavage rate, blastocyst formation rate and cell numbers. The oocytes from the 5, 20 and 90 min co-incubation periods were washed in IVF medium (mTBM) to remove spermatozoa, transferred to another droplet of the same medium (containing no spermatozoa) and incubated until 6 h post-insemination. Thereafter, all IVF embryos were cultured in porcine zygote medium-3 for 10 h after insemination to assess fertilization parameters. A co-incubation period of 5 min resulted in a significantly ( $p < 0.05$ ) lower spermatozoa penetration rate (66.8%) as compared with 20, 90 and 360 min (91.8, 100 and 100%, respectively) but there was no difference in Male Pronucleus (MPN) formation rate. Co-incubation for 5 and 20 min resulted in a significantly higher monospermy rate (39.7 and 38.4%, respectively) as compared with the 90 min incubation (4.2%) while no monospermic oocytes were observed in the 360 min group. The polyspermy rate was significantly higher with co-incubation periods of 90 and 360 min (95.8 and 100%, respectively) as compared with 5 and 20 min (60.3 and 57.8%, respectively). The mean number of penetrating spermatozoa per oocyte was significantly lower in the 5 and 20 min groups (1.7 and 2.3, respectively) as compared with the 90 and 360 min groups (9.2 and 17.6, respectively). The efficiency of fertilization (number of monospermic oocytes/total number of inseminated oocytes) was significantly higher in the 20 min (35.8%) co-incubation period as compared with the other groups (25.1 and 4.2% for 5 and 90 min, respectively). The cleavage rate for the 5 and 20 min (52.5 and 65.6%, respectively) groups was significantly higher than the 90 and 360 min groups (32.1 and 10.5%, respectively). The 20 min (18.8 and 113.2%) co-incubation period resulted in a significantly higher blastocyst formation rate and total blastocyst cell number compared to the 90 min group (3.6 and 68.0%) but there was no significant difference between the 5 min (10.7 and 90.3%) and 20 min groups. The embryos in the 360 min co-incubation period did not develop into blastocysts. Concerning cell numbers, blastocysts that developed with the 20 min (24.6) co-incubation period had a greater number of cells in the inner cell mass than the 5 min (12.4) group however, there was no difference in the number of trophectoderm cells and total cell number. Therefore, researchers recommend a two-step IVF procedure for porcine oocytes with a co-incubation period with spermatozoa for 20 min followed by an additional culture until 6 h without spermatozoa when using commercial LS semen for porcine IVF experiments.

**Key words:** Co-incubation period, commercial liquid stored semen, IVF, polyspermy, porcine embryo

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### INTRODUCTION

Over the past decade, mammalian embryos have been routinely cultivated *in vitro* up to the blastocyst stage for use in numerous studies including as biomedical models

for human physiology and diseases and for xenotransplantation (Niemann and Rath, 2001; Nusuetrong *et al.*, 2010). Porcine embryos matured and fertilized *in vitro* can develop to the blastocyst stage *in vivo* (Mattioli *et al.*, 1989; Funahashi *et al.*, 1994) and

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**Corresponding Author:** Sang-Hwan Hyun, Laboratory of Veterinary Embryology and Biotechnology,  
College of Veterinary Medicine, Chungbuk National University, 361-763 Cheongju, Republic of Korea

*in vitro* (Rath *et al.*, 1995; Abeydeera and Day, 1997a; Machaty *et al.*, 1998). Although, techniques for *In Vitro* Maturation (IVM) and *In Vitro* Fertilization (IVF) for porcine oocytes have been developed in recent years, the high incidence of polyspermic penetration is still a major obstacle to the successful production of a large number of porcine embryos *in vitro* (Funahashi and Day, 1997; Day, 2000; Abeydeera, 2002; Coy and Romar, 2002; Funahashi, 2003). Many studies have been conducted in an attempt to decrease the incidence of polyspermy *in vitro*, simulating the *in vivo* conditions. Some unique porcine IVF strategies such as the straw-IVF method (Li *et al.*, 2003; Alminana *et al.*, 2008a), the climbing over the wall method (Funahashi and Nagai, 2000) or microfluidic technology (Beebe *et al.*, 2002; Clark *et al.*, 2003, 2005) have been reported in an attempt to mimic the process of *in vivo* fertilization in the oviductal tract.

Several studies suggest that longer spermatozoa-oocyte co-incubation periods are associated with higher penetration rates and higher levels of polyspermy (Coy *et al.*, 1993; Martinez *et al.*, 1996; Abeydeera and Day, 1997b; Marchal *et al.*, 2002; Gil *et al.*, 2007). In fact, most of the recent microdrop-IVF systems use a 5-6 h spermatozoa-oocyte co-incubation period when using frozen-thawed semen (Abeydeera and Day, 1997b; Funahashi *et al.*, 1999; Wang *et al.*, 1999; Gil *et al.*, 2003) as compared with the 12-18 h used in the original porcine IVF systems (Iritani *et al.*, 1978). In contrast, it was reported that there are sufficient spermatozoa bound to the Zona Pellucida (ZP) within the 1st 2-10 min of spermatozoa-oocyte co-incubation to penetrate a high number of oocytes (Gil *et al.*, 2004, 2007; Alminana *et al.*, 2008b). Therefore, a short co-incubation time was suggested as an alternative method to increase the efficiency of IVF with porcine oocytes (Gruppen and Nottle, 2000; Funahashi and Romar, 2004; Gil *et al.*, 2004; Alminana *et al.*, 2005).

In many porcine IVF experiments, frozen-thawed sperm is used because it provides reproducible data and the possibility of repeating experiments. However, frozen-thawed sperm is damaged in cryopreservation and sperm freezing and thawing was reported to result in a reduction in viability, changes in sperm function, lipid composition and organization of the sperm plasma membrane (Buhr *et al.*, 1994). Despite the different characteristics between frozen-thawed sperm and fresh sperm, some studies (Koo *et al.*, 2000; Lee *et al.*, 2010) have conducted porcine IVF using fresh sperm in the conventional procedure (microdrop-IVF systems; 6 h spermatozoa-oocyte co-incubation). In this point of view, researchers also conducted porcine IVF using fresh sperm derived from commercial Liquid Stored (LS) semen for some

experiments using the conventional method. Interestingly, using this IVF procedure researchers obtained results showing that a large number of spermatozoa bound to the ZP and most of the oocytes did not cleave and were arrested in the 1 cell stage (same as fragmentation pattern 1 reported by Mateusen *et al.*, 2005).

Based on the laboratory results and the findings, this study was designed to investigate the appropriate spermatozoa-oocyte co-incubation time for the porcine IVF system using fresh sperm to decrease polyspermic fertilization and improve the *in vitro* production of porcine blastocysts. In this study, different spermatozoa-oocyte co-incubation times (5, 20, 90 and 360 min) were tested to determine the effect on fertilization parameters. To test developmental competence, porcine oocytes matured *in vitro* were fertilized with variable co-incubation times and then cultured *in vitro* for 7 days after which time the embryos were assessed at the blastocyst stage of development. In addition, the cell numbers in blastocysts that developed in the experimental groups were examined to evaluate embryo quality.

## MATERIALS AND METHODS

**Chemicals:** Unless otherwise stated, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO).

**Oocytes collection and *in vitro* maturation:** Ovaries of prepubertal gilts were collected from a local slaughterhouse and transported to the laboratory within 1 h in physiological saline supplemented with 100 IU mL<sup>-1</sup> penicillin G and 100 mg mL<sup>-1</sup> streptomycin sulfate at 30-35°C. The Cumulus-Oocyte Complexes (COCs) were aspirated using an 18 gauge needle attached to a 10 mL disposable syringe (Kim *et al.*, 2004) from superficial follicles 3-6 mm in diameter and pooled in to 15 mL conical tubes and allowed at 37°C during 5 min to settle down as sediment. The supernatant was discarded and the precipitate was resuspended with HEPES-buffered Tyrode's medium (TLH) containing 0.05% (w/v) Polyvinyl Alcohol (TLH-PVA) and observed under a stereomicroscope. Only compact COCs with ≥3 uniform layers of compact cumulus cells and homogenous cytoplasm were recovered from the collected fluid and washed three times in TLH-PVA. Approximately 50-60 COCs were transferred into each well of a 4-well Nunc dish (Nunc, Roskilde, Denmark) containing 500 µL of culture medium (TCM-199; Invitrogen Corporation, Carlsbad, CA, USA) which was supplemented with 0.6 mM cysteine, 0.91 mM sodium pyruvate, 10 ng mL<sup>-1</sup> epidermal growth factor, 75 µg mL<sup>-1</sup> kanamycin, 1 µg mL<sup>-1</sup> insulin, 10% (v/v) porcine Follicular Fluid

(pFF), fresh 10 IU mL<sup>-1</sup> equine Chronic Gonadotropin (eCG) and 10 IU mL<sup>-1</sup> hCG (Intervet, Boxmeer, Netherlands). The pFF was aspirated from 3-7 mm follicles of prepubertal gilt ovaries. After centrifugation at 1600×g for 30 min, the supernatants were collected and filtered sequentially through 1.2 and 0.45 µm syringe filters (Gelman Sciences, Ann Arbor, MI, USA). The prepared pFF was then stored at -20°C until use. For maturation, the selected COCs were incubated at 39°C in a humidified atmosphere of 5% CO<sub>2</sub> in 95% air. After 21-22 h of maturation with hormones, the COCs were washed two times in a maturation medium without hormone supplements and then cultured for 21-22 h without hormone supplements at 39°C in a humidified atmosphere of 5% CO<sub>2</sub> in 95% air.

**In vitro fertilization and culture:** For *In Vitro* Fertilization (IVF), commercial Liquid Stored (LS) semen produced for artificial insemination was supplied weekly from the Veterinary Service Laboratory (Department of Livestock Research, Yong-in city, Gyeonggi-do, Republic of Korea), it was diluted in the Androhep® (Minitube, Germany) boar semen extender and kept at 17°C for 5 days before use. Semen sample was washed two times by centrifugation with Dulbecco's Phosphate Buffered Saline (DPBS) supplemented with 0.1% BSA at 2000×g for 2 min. After washing, the sperm pellet was re-suspended in modified Tris-Buffered Medium (mTBM) (Abeydeera and Day, 1997a) which was pre-equilibrated for 18 h at 39°C under 5% CO<sub>2</sub>. After 42-44 h of IVM, the COCs were denuded by gently pipetting with 0.1% hyaluronidase and washed three times in TLH-PVA. Oocytes with evenly ooplasm and visible first polar body were used for all experiments. Groups of 15 oocytes were randomly placed into 40 µL droplets of mTBM in a 35×10 mm Petri dish (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA) covered with pre-warmed mineral oil. After appropriate dilution, 5 µL of the sperm suspension was added to a 40 µL drop of fertilization medium (mTBM) to yield a final sperm concentration of 5×10<sup>5</sup> sperms mL<sup>-1</sup> (1667 spermatozoa/oocyte). Just before fertilization, sperm motility was assessed by placing a drop of sperm suspension on a warm slide glass and examining it subjectively at 200× magnification and >70% motile sperms were used in every experiment. The oocytes were co-incubated with spermatozoa at 39°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air for 5, 20, 90 and 360 min. After co-incubation with sperms, the loosely attached sperms were removed from the ZP by gentle pipetting. The oocytes from 5, 20 and 90 min of co-incubation periods were washed three times in IVF medium (mTBM) and transferred to another droplet of the same medium (containing no spermatozoa) and incubated until 6 h post insemination. Thereafter, gametes were

washed three times with embryo culture medium and cultured in 25 µL microdrops (10 gametes/drop) of Porcine Zygote Medium (PZM)-3 (Yoshioka *et al.*, 2002). The embryos with cultured drop were covered with pre-warmed mineral oil and incubated at 39°C for 168 h under a humidified atmosphere of 5% O<sub>2</sub>, 5% CO<sub>2</sub> and 90% N<sub>2</sub>. In all experiments, the culture media were renewed at 48 h (day 2) and 96 h (day 4) after IVF.

**Evaluation of sperm penetration and pronuclear formation:** Fertilization parameters and pronuclear formation was performed according to Koo *et al.* (2005) with some modifications. Briefly, at 10 h after insemination, the ZP of oocytes was dissolved with 0.5% pronase. The zona-free embryos were washed three times in TL-HEPES medium containing 0.1% formaldehyde and 0.01% PVA for 1 min and fixed in 1% formaldehyde and 0.01% PVA in PBS for 10 min at room temperature. The fixed embryos were placed in a drop of mounting medium [25% (v/v) glycerol in PBS containing 2.5 mg mL<sup>-1</sup> sodium azide and 2.5 µg mL<sup>-1</sup> Hoechst 33342] on a slide and a cover slide was placed over the embryos gently. The number of spermatozoa penetrating the ZP (percentage of the number of penetrated oocytes/total inseminated), Male Pronuclear formation (MPN), the presence of monospermy (percentage of the number of monospermic oocytes/total penetrated), polyspermy (percentage of the number of polyspermic oocytes/total penetrated), number of penetrated spermatozoa/oocyte (mean number of spermatozoa in penetrated oocytes) and efficiency of fertilization (number of monospermic oocytes/total inseminated) were examined under a fluorescence microscope.

**Total cell count and differential staining of blastocysts:** To determine the total cell number of blastocysts, at 168 h after IVF (day 7), blastocysts were collected and washed in 1% (w/v) PBS-BSA and stained with 10 µg mL<sup>-1</sup> Hoechst-33342 (bisbenzimidazole) for 5 min. Following a final wash in PBS-BSA, embryos were fixed briefly in 4% paraformaldehyde in PBS. Then the blastocysts were mounted on glass slides in a drop of 100% glycerol, squashed gently with a cover slip and observed under a fluorescence microscope (Nikon Corp., Tokyo, Japan) at 400× magnification.

The quality of the blastocysts was assessed by the differential staining of Inner Cell Mass (ICM) and Trophectoderm (TE) cells according to a modified staining procedure (Thouas *et al.*, 2001). Briefly, TE cells of blastocysts at 168 h were stained with 100 µg mL<sup>-1</sup> fluorochrome propidium iodide after treatment with permeabilizing solution containing 1% (v/v) Triton X-100 ionic detergent. Blastocysts were then incubated in a

second solution containing 100% ethanol (for fixation) at 4°C and bisbenzimidazole. Fixed and stained whole blastocysts were mounted and assessed for the number of cells present using epifluorescence microscopy (Leica, Germany) at 400× magnification.

**Statistical analysis:** The statistical analysis was conducted using the SPSS Inc. Software (PASW-17 Statistical Software). The means of the penetration rate, MPN formation rate, monospermic rate, polyspermic rate, efficiency data, cleavage rate, developmental rate to the blastocyst stage and number of nuclei were compared by one-way ANOVA followed by Duncan's multiple range test. Data were presented as mean±SEM. Differences were considered to be significant if the  $p < 0.05$ .

## RESULTS AND DISCUSSION

To determine if the efficiency of *in vitro* production of porcine embryos using LS semen could be improved by a reduction in the co-incubation time, a total of 808 oocytes were inseminated from which 343 were fixed and stained to assess fertilization parameters and 465 were cultured for 7 days to assess embryo development. The effects of spermatozoa-oocyte co-incubation times on spermatozoa penetration rate were examined using a spermatozoa concentration of  $5.0 \times 10^5$  cells  $\text{mL}^{-1}$  (1667 spermatozoa/oocyte) (Abeydeera *et al.*, 1998). As shown in Table 1, co-incubation times significantly affected ( $p < 0.05$ ) the penetration rate, monospermy, polyspermy, the number of penetrated spermatozoa/oocyte and the efficiency of fertilization. A co-incubation time of 5 min resulted in a significantly ( $p < 0.05$ ) lower spermatozoa penetration rate (66.8%) as compared with 20, 90 and 360 min (91.8, 100 and 100%, respectively) but no

difference in MPN formation rate (Table 1). Co-incubation times of 5 and 20 min resulted in a significantly higher monospermy rate (39.7 and 38.4%, respectively) as compared with the 90 min (4.2%) co-incubation time and no monospermic oocytes were observed with a 360 min co-incubation time. The polyspermy rate was significantly higher with co-incubation times of 90 and 360 min (95.8 and 100%, respectively) as compared with 5 and 20 min (60.3 and 57.8%, respectively). The mean number of penetrating spermatozoa per oocyte was significantly lower in the 5 and 20 min groups (1.7 and 2.3, respectively) than the 90 min (9.2) and 360 min (17.6) groups (Table 1 and Fig. 1). The efficiency of fertilization was significantly higher in the 20 min (35.8%) co-incubation group as compared with the other groups. The 5 min (25.1%) co-incubation time had a lower efficiency of fertilization than the 20 min group but was still higher than in the 90 min group (4.2%) (Table 1).

To determine whether co-incubation time influences *in vitro* developmental competence, porcine embryos derived from each experiment were cultured *in vitro* for 7 days and then the cleavage rate, blastocyst formation and total blastocyst cell number were observed. The cleavage rate in the 5 and 20 min (52.5 and 65.6%, respectively) groups was significantly higher than in the other groups. In addition, the cleavage rate for the 90 min group (32.1%) was significantly less than 5 and 20 min groups but >360 min group (10.5%) (Table 2). The 20 min (18.8 and 113.2%) co-incubation time resulted in a significantly higher blastocyst formation rate and total blastocyst cell number as compared with the 90 min group

Table 1: Effect of the duration of spermatozoa-oocyte co-incubation on sperm penetration of *in vitro* matured porcine oocytes at 10 h post insemination

	Co-incubation time (min)			
Sperm penetration	5	20	90	360
No. of oocytes examined	75	96	88	84
Penetrated (%) <sup>*</sup>	66.8±5.7 <sup>a</sup>	91.8±2.0 <sup>b</sup>	100±0 <sup>b</sup>	100±0 <sup>b</sup>
MPN formed (%) <sup>**</sup>	93.3±6.7	94.2±2.1	98.7±1.3	99.1±0.9
Monospermy (%) <sup>**</sup>	39.7±6.4 <sup>a</sup>	38.4±4.5 <sup>a</sup>	4.2±1.4 <sup>b</sup>	NO
Polyspermy (%) <sup>**</sup>	60.3±6.4 <sup>a</sup>	57.8±3.7 <sup>a</sup>	95.8±1.4 <sup>b</sup>	100±0 <sup>b</sup>
Sperm/penetrated	1.7±0.3 <sup>a</sup>	2.3±0.2 <sup>a</sup>	9.2±0.7 <sup>b</sup>	17.6±2.1 <sup>c</sup>
Efficiency of fertilization (%) <sup>†</sup>	25.1±3.1 <sup>a</sup>	35.8±3.3 <sup>b</sup>	4.2±1.4 <sup>c</sup>	NO

Data are given as mean±SEM; <sup>\*</sup>Percentage of the number of oocytes examined; <sup>\*\*</sup>Percentage of the number of oocytes penetrated; <sup>†</sup>Efficiency of fertilization was the percentage of monospermic oocytes from total examined; <sup>a-c</sup>Values with different superscripts within rows are significantly different ( $p < 0.05$ ), experiment was repeated six times; MPN: Male Pronucleus; NO: Not Observed

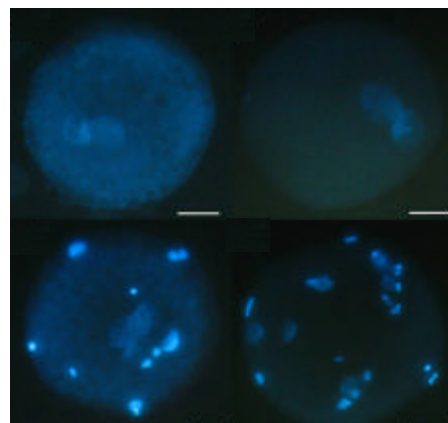


Fig. 1: Porcine oocytes which were 10 h post insemination stained with Hoechst 33342 and observed under an epifluorescence microscope. a-d represent stained zygotes produced from 5, 20, 90 and 360 min co-incubation time, respectively. Bar = 25  $\mu\text{m}$

Table 2: Effect of different spermatozoa-oocyte co-incubation times during IVF on the development of porcine embryos *in vitro*

Co-incubation time (min)	No. of oocytes	No. of cleaved embryos (%)	No. of blastocysts (%)	Total cell number (n)*
5	133	71 (52.5±5.4) <sup>a</sup>	15 (10.7±3.9) <sup>a,b</sup>	90.3±12.7 <sup>a,b</sup> (n = 9)
20	120	78 (65.6±3.9) <sup>a</sup>	24 (18.8±5.1) <sup>b</sup>	113.2±6.7 <sup>b</sup> (n = 13)
90	108	30 (32.1±9.8) <sup>b</sup>	3 (3.6±2.4) <sup>a</sup>	68.0±7.8 <sup>a</sup> (n = 3)
360	104	9 (10.5±3.3) <sup>c</sup>	NO	NO

\*Number of examined blastocyst; <sup>a,b</sup>Values with different superscripts within a column differ significantly ( $p < 0.05$ ), experiment was repeated four times; data are given as mean±SEM. NO: Not Observed

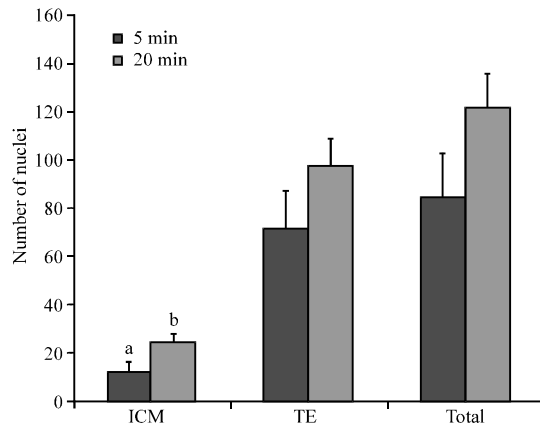


Fig. 2: Cell numbers of different co-incubation time (5 vs. 20 min) derived blastocysts that developed *in vitro*. Number of ICM cell of 20 min co-incubation time derived blastocysts were significantly ( $p < 0.05$ ) > 5 min co-incubation time derived blastocysts but no differences were detected in the number of TE or total cell

(3.6 and 68.0%) however, there was no difference between 5 min (10.7% and 90.3) and 20 min. The embryos in the 360 min co-incubation group did not develop into blastocysts. Blastocysts that developed in the 20 min (24.6) co-incubation group had a greater number of cells in the Inner Cell Mass (ICM) than the 5 min (12.4) group but there was no difference in the number of Trophectoderm (TE) cells or total cell number (Fig. 2 and 3). Additionally, the ratio of ICM cells per TE cell was significantly higher in the 20 min co-incubation group as compared with the 5 min group ( $0.26 \pm 0.03$  vs.  $0.18 \pm 0.02$ , respectively).

The high incidence of polyspermic penetration is still a major obstacle for successful production of a large number of porcine embryos *in vitro* (Funahashi and Day, 1997; Day, 2000; Abeydeera, 2002; Coy and Romar, 2002; Funahashi, 2003). Low efficiency in porcine IVF, related mainly to the high incidence of polyspermic penetration has lead to the development of new IVF strategies. Some of these include changes in both the IVF system (Funahashi and Nagai, 2000; Beebe *et al.*, 2002; Li *et al.*, 2003; Clark *et al.*, 2005; Alminana *et al.*, 2008a) and spermatozoa-oocyte co-incubation periods

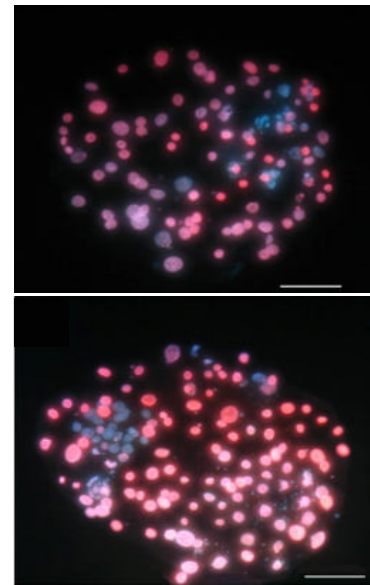


Fig. 3: Differentially stained day 7 porcine blastocysts. The blue objects are the nuclei of ICM cells and the red objects are the nuclei of TE cells; a) differentially stained blastocyst produced from 5 min co-incubation time and b) differentially stained blastocyst produced from 20 min co-incubation time. The nuclei of blastocysts were photographed under magnification of 400× and using a fluorescence microscope. Bar = 50 μm

(Funahashi and Romar, 2004; Gil *et al.*, 2007, 2004; Alminana *et al.*, 2005). These procedures have concentrated on reducing the exposure of oocytes to the number of spermatozoa reaching the surface and the spermatozoa-oocyte co-incubation time. Several studies have investigated decreasing the rate of *in vitro* polyspermy by modifying the spermatozoa-oocyte co-incubation periods for porcine IVF (Coy *et al.*, 1993; Gil *et al.*, 2004, 2007). It was reported that there are enough spermatozoa bound to the ZP within 10 min of spermatozoa-oocyte co-incubation to penetrate a high number of oocytes (Gil *et al.*, 2004, 2007; Alminana *et al.*, 2008b). Moreover, good penetration rates have been reported in human IVF using only 30 sec of co-incubation (Bungum *et al.*, 2006). However, these studies were conducted using frozen-thawed semen

and few studies have been conducted using fresh semen but the period of spermatozoa-oocyte co-incubation depends on the laboratory (Koo *et al.*, 2000; Funahashi and Romar, 2004; Koo *et al.*, 2005; Lee *et al.*, 2010). Some experiments (Koo *et al.*, 2000; Lee *et al.*, 2010) of porcine IVF using fresh semen conducted same as conventional procedures (6 h co-incubation in microdrops) despite of different characteristics between frozen-thawed semen and fresh semen.

In the present study, researchers conducted an IVF experiment using commercial LS semen (fresh semen) because the cryopreserved boar semen made in the laboratory was contaminated by antibiotic resistant bacteria (*Enterobacter cloacae*) derived from egg yolk (Jang *et al.*, 2011) and researchers were unable to use it. Cryopreserved sperm is damaged during freezing and thawing and sperm cryopreservation was reported to result in a reduction in viability, changes in sperm function, lipid composition and organization of the sperm plasma membrane (Buhr *et al.*, 1994). In addition, cryopreserved boar spermatozoa have been observed to show a capacitation-like reaction (Watson, 1995; Green and Watson, 2001) and some damage to the acrosome and flagella regions (Courtens and Paquignon, 1985). All of these findings encouraged us to investigate using porcine LS semen in order to determine whether short co-incubation periods would be satisfactory in achieving good penetration rates thus, reducing the incidence of polyspermic fertilization and to use it as alternative method for porcine IVF when frozen-thawed semen cannot be used. For this reason, researchers reduced the spermatozoa-oocyte co-incubation time from 6 h to 90, 20 and 5 min. In the current study, researchers used a concentration of  $5 \times 10^5$  cells  $\text{mL}^{-1}$  (1667 spermatozoa/oocyte) because a previous report (Gil *et al.*, 2007) showed that the efficiency of IVF was best with a short (10 min) co-incubation combined with high spermatozoa/oocyte ratios (1500 spermatozoa/oocyte). Based on the findings, the appropriate spermatozoa-oocyte co-incubation period is 20 min in terms of the efficacy of fertilization, blastocyst formation rate and cell number in the ICM when LS semen is used. The results demonstrate that the penetration rates were significantly higher with co-incubation times of 20, 90 and 360 min than with 5 min however, the 90 and 360 min groups displayed higher polyspermy rates (96.0 and 100.0%, respectively) and contained significantly more penetrating sperm than the 5 and 20 min groups. The efficiency of fertilization in the 90 min group was significantly less than 5 and 20 min groups and we could not find a monospermic embryo in the 360 min co-incubation group. These results demonstrate that a co-incubation time of 6 h (as in the

conventional microdrop IVF procedure) is not suitable for porcine IVF using fresh semen. Researchers demonstrated that the incidence of polyspermic penetration could be decreased by shortening the duration of co-incubation. These results are consistent with previous studies with both frozen-thawed (Gil *et al.*, 2004) and fresh spermatozoa (Funahashi and Romar, 2004) showing that shortening the co-incubation period to 5-20 min decreases the incidence of polyspermy. When the spermatozoa-oocyte co-incubation period is extended from 20-360 min (6 h), the proportion of penetrated oocytes and the number of spermatozoa per oocyte increases gradually over time (Abeydeera and Day, 1997b; Marchal *et al.*, 2002) because spermatozoa-oocyte interactions are higher. However when oocytes are washed after 20 min of co-incubation and then transferred to a medium without spermatozoa, new interactions are prevented but ZP-bound spermatozoa are able to penetrate the oocytes. Although, it has been considered that the lower oxidative stress (caused by reactive oxygen species) suffered by the gametes could explain the higher incidence of polyspermic penetration observed after short-term co-incubation (Gil *et al.*, 2004), more experiments are needed to explain this phenomenon. The results showed that LS spermatozoa bound to the oocytes for only 5 min after insemination had a polyspermic fertilization rate of about 51.5%. Polyspermy rates even with short (5-20 min) co-incubation periods appear to be affected by the donor of the oocytes (Marchal *et al.*, 2001) and are still higher for *in vitro* matured oocytes than for ovulated oocytes (Wang *et al.*, 1998). Improvement of the quality of porcine IVM oocytes and the use of such oocytes in the current IVF system may help to reduce polyspermy.

Gil *et al.* (2008) examined fertilization parameters obtained under various short co-incubation periods, types of spermatozoa and spermatozoa:oocyte ratios in IVF experiments. In their report, for the short (10 min) co-incubation time for porcine IVF using frozen-thawed sperm, the penetration rate was approximately 60~70% and the efficiency rate was around 40%. Compared to the findings, the penetration rate (91.8%) for the 20 min co-incubation time is >10 min co-incubation time using frozen-thawed sperm but the efficiency of fertilization (35.8%) is nearly the same. The lower penetration with the short (10 min) co-incubation time using frozen-thawed sperm might be due to sperm damage during cryopreservation. Previous studies also examined IVF using fresh semen and different spermatozoa-oocyte co-incubation periods (Funahashi and Romar, 2004; Koo *et al.*, 2005) however, the results differed as compared with the experiment. In the IVF experiment

of Koo *et al.* (2005) 2, 4 and 6 h spermatozoa-oocyte co-incubation times with a concentration of  $1.2 \times 10^5$  cells  $\text{mL}^{-1}$  were examined. The penetration rates were 68.8, 80.3 and 89.9% for 2, 4 and 6 h, respectively and there was no difference in polyspermy, MPN formation and mean number of penetrating spermatozoa per oocyte (approximately 1.4). These results are quite different from the findings. In the IVF experiment of Funahashi and Romar (2004) using 5, 10, 15 and 30 min co-incubation periods, the penetration rate at 5 min (91.9%) was higher than the result (67.9%) but the monospermy rates were lower (26.5 and 4.0% at 5 and 15 min, respectively) than the findings (39.7 and 38.4% at 5 and 20 min, respectively). The reasons for this discrepancy might be due to the different IVF medium (Funahashi *et al.*, 1994), the different semen source (boar effect) (Alminana *et al.*, 2005) or the different concentration of spermatozoa used in these studies. Koo *et al.* (2005) used  $1.2 \times 10^5$  cells  $\text{mL}^{-1}$  and Funahashi and Romar (2004) used  $2.5 \times 10^5$  cells  $\text{mL}^{-1}$ .

Researchers investigated whether the spermatozoa-oocyte co-incubation time influences *in vitro* developmental competence. The cleavage rate was significantly higher in the 5 and 20 min groups as compared with the 90 and 360 min groups. With 360 min co-incubation, researchers obtained few (10.5%) cleaved embryos. A co-incubation time of 20 min resulted in higher blastocyst formation and total blastocyst cell numbers. The cell numbers in the ICM and TE of the blastocyst were also compared between the 5 and 20 min groups but researchers could not obtain enough blastocyst samples for differential staining with the 90 and 360 min co-incubation periods. Blastocysts that developed from the 20 min co-incubation group had greater ICM cell numbers than the 5 min group but no differences in TE and total cell number. The relative numbers of blastocyst ICM and TE cells can influence the developmental competence of the fetus after implantation (Machaty *et al.*, 1998). Thus, the higher numbers of ICM cells in the 20 min group may be the reason for the higher fertilization efficiency as compared with the 5 min group. In the 90 min co-incubation group, the total blastocyst cell number was significantly <20 min group. These results indicate that a long exposure to spermatozoa during IVF induces high polyspermic penetration.

Additionally, live and dead sperm may also be detrimental to oocytes thus affecting subsequent embryo development. It was shown that the developmental potential of polyspermic porcine embryos to the blastocyst stage is similar to that of normal embryos but that developmental competence to term is much lower. Previous studies have indicated that polypronuclear oocytes produced from IVF develop to the blastocyst

stage *in vitro* or *in vivo* at a similar rate to two-pronuclear oocytes (Han *et al.*, 1999a, b; McCauley *et al.*, 2003). A previous study demonstrated that blastocysts produced from polyspermic-derived porcine embryos had lower ICM cell numbers than monospermic-derived embryos although, no difference was detected in the number of TE cells between the two groups (Han *et al.*, 1999a). Polyspermy causes aberrant development and early embryo death (Hunter, 1991).

## CONCLUSION

In conclusion, the study demonstrated that under *in vitro* conditions, 20 min of spermatozoa-oocyte co-incubation during porcine IVF using LS semen improves the efficiency of fertilization and *in vitro* porcine embryo production. When frozen-thawed semen is not available for porcine IVF, commercial LS semen can be used as an alternative. Researchers suggest a two-step IVF procedure for porcine oocytes with a co-incubation with spermatozoa for 20 min followed by an additional culture without spermatozoa for 6 h when using LS semen.

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