Effects of *In Vitro* Fertilization Conditions of *In Vitro* Matured Cumulus-Intact Pig Oocytes on Embryo Development

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ABSTRACT

In this study, we examined the effectiveness of *in vitro* fertilization of porcine immature oocytes on the embryo development of blastocysts or hatched blastocysts and the number of cells according to the *in vitro* fertilization conditions. In the *in vitro* fertilization of *in vitro* matured porcine oocytes, there were no significant differences between treatment groups regarding fertilization rate, blastocyst rate, and embryo development of hatched blastocysts according to the storage periods of liquid sperm of 24, 48, and 72 hours. The embryo development rate of hatched blastocysts after the fertilization according to different spermatozoa concentrations (0.4×10^5, 1.2×10^5, and 3.6×10^5 cells/ml) showed the highest rate in the group with a spermatozoa concentration of 1.2×10^5 cells/ml; in particular, this rate was significantly higher than that in the 0.4×10^5 cells/ml group ($p<0.05$). The total number of blastocysts cells as well as trophectoderms (TE) that developed in each treatment group were also significantly higher in the 1.2×10^5 cells/ml group than in any other groups ($p<0.05$). In contrast, the embryo development rate of blastocysts according to different co-incubation periods of sperm and oocyte (1, 3, and 6 hr) was high in the 6-hour group; in particular, the rate was significantly higher than that of the 1-hour group ($p<0.05$). Furthermore, the total number of oocytes cells and TEs that developed was significantly higher in the 6-hour group than any other group ($p<0.05$).

In this study, the most effective treatment conditions for porcine embryo development and high cell number were found to be as follows: a sperm storage period of less than 72 hours, a spermatozoa concentration of 1.2×10^5 cells/ml, and a 6-hour co-incubation period for sperm and ooocyte.

(Key words : porcine, blastocysts, spermatozoa)

INTRODUCTION

The production techniques used to produce *in vitro* embryos have been used to investigate mammalian embryogenesis; furthermore, these techniques have improved the quality of human life by transforming animal production (Hansel and Godke, 1992), facilitating the developing oophyte micromanipulation, and furthering stem cell research. However, until now, these techniques have been limited to laboratory animals, and have only rarely been used for livestock (Ebert and Schindler, 1993).

Since porcine fertilization was successfully accomplished by Iritani *et al.* (1978), several *in vitro* fertilization studies using porcine immature oocytes have been performed. Supplementation of oocytes with caffeine to enhance fertilization by sperm (Flesch and Gadella, 2000) and diverse sperm treatment methods (Matas, 2003) have been studied and there have been several reports on how excessive amounts of sperm as well as fertilization times increase the probability of polyspermic penetration (Abeydeera and Day, 1997; Marchal *et al.*, 2002; Matas *et al.*, 2003). Specifically, the relative number of inner cell mass (ICM) of blastocyst or trophectoderm (TE) cells can be used to assess the quality of blastocysts. Interestingly, the blastocyst cell number after polyspermic penetration decreased compared to normal samples (Giles and Foote, 1995).

Likewise, polyspermic penetration into *in vitro* matured porcine oocytes causes deterioration in the quality of *in vitro*-produced blastocysts. It has been reported that problems associated with polyspermic penetration can be solved by reducing the number of sperm per ovum in the culture medium (Nagai and Moor, 1990). However, the less sperm, the lower the polyspermic penetration rate, which in turn can reduce the overall *in vitro* fertilization rate (Abeydeera and Day, 1997).

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Therefore, despite the many previous studies, the problem of polyspermy remains unsolved. To address this issue, we counted the number of cells to assess embryo development and quality according to the storage period of liquid sperm, various spermatozoa concentrations, and the co-incubation period of sperm and oocyte.

MATERIAL AND METHODS

1. Chemicals and Media

Unless otherwise stated, all chemicals used in this study were purchased from Sigma Chemical Co. (St, Louis, MO, USA). Solutions are expressed as percent dilutions (v:v) and all media used for IVM, IVF, and *in vitro* culture (IVC) were pre-warmed to 39℃ in a 5% CO_2 incubator with maximum humidity for 4 h before use.

2. Oocyte Collection and *In Vitro* Maturation

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in saline supplemented with 25 μg/mL gentamicin at 25~30℃ within 2~3 hours. Cumulus oocyte complexes (COCs) were obtained by aspiration from follicles 2 to 6 mm in diameter using an 18-gauge needle connected to a 10-ml disposable syringe. Only COCs with compact cumulus cell layers and evenly granulated ooplasm were selected. The COCs were washed three times in HEPES-Tyrode-albumin-lactate-pyruvate medium (TALP medium) supplemented with 25 mM HEPES and 3 mg/ml BSA. Groups of 50 COCs were placed into 500 μl of BSA-free NCSU-23 solution with 0.57 mM cysteine, 10% porcine follicular fluid (pFF), 2.5 mM β-mercaptoethanol, 10 ng/ml epidermal growth factor (EGF), 10 IU/ml human chorionic gonadotropin (hCG) and 10 IU/ml pregnant mare serum gonadotropin (PMSG) in each well of a 4-well multidish (Nunc, Roskilde, Denmark). After 22 h for maturation, oocytes were washed twice in the same maturation medium without PMSG and hCG and cultured in this medium for 22 h at 39℃ in an atmosphere of 5% CO_2 and maximum humidity.

3. Sperm Preparation and *In Vitro* Fertilization

Diluted porcine semen was obtained from the Dabby A.I. Center and stored at 17℃ for 24, 48, or 72 hr. The semen was layered on top of a discontinuous Percoll density gradient (2 ml of 45% Percoll over 2 mL of 90% Percoll) in a 15-ml centrifuge tube. The sample was centrifuged for 20 min at 500 × g at room temperature. The spermatozoa collected in the bottom fraction were washed three times: twice in Dulbecco's phosphate-buffered saline (D-PBS, Gibco, USA) containing 1 mg/mL BSA, 100 μg/ml penicillin, and 75 μg/ml streptomycin at 500 × g for 5 minutes, and once in mTBM. The spermatozoa were diluted with mTBM to obtain the desired final concentration.

After the IVM period, oocytes were briefly treated with 0.1% hyaluronidase in Dulbecco's D-PBS supplemented with 1 mg/mL BSA to remove cumulus cells, and were washed 2~3 times with modified Tris-buffered medium (mTBM) containing 1 mg/ml BSA and 2.5 mM caffeine sodium benzoate. After washing, groups of 25~30 oocytes were placed in 48-μl droplets of mTBM in 60-mm petri dishes that had been covered with warm mineral oil. Two microliters of spermatozoa suspension were added to each fertilization drop, resulting in a final concentration of 0.4×10^5, 1.2×10^5, or 3.6×10^5 spermatozoa/ml. Oocytes and spermatozoa were co-incubated for 1, 3, or 6 hr at 39℃ and 5% CO_2 with maximum humidity.

4. *In Vitro* Culture

We washed each of the presumptive zygotes (day 0) treated with *in vitro* fertilization conditions with TL-HEPES solution three times and removed impurities, including sperm, and then washed the zygotes two or three times with PZM3 solution. Next, we inserted 30~40 oocytes into 50 μl of PZM3 solution and cultured them at 39℃ with 5% CO_2 to enhance *in vitro* development. Forty-eight hours after *in vitro* culture was initiated, we investigated the rate of *in vitro* fertilization of fertilized oocytes and then, 168 hours later, we examined the embryo development of blastocysts or hatched blastocysts.

5. Blastocyst Differential Staining

The zona pellucida of the blastocysts was removed with a 0.5% protease solution and washed 4~5 times in TL-HEPES solution with 0.1% PVA (TL-PVA). Zona-free blastocysts were incubated in a 1:5 dilution of rabbit anti-bovine whole serum in TL-PVA medium for 1 h. After being washed five more times in TL-PVA medium, blastocysts were re-incubated in a 1:10 dilution of a guinea pig complement in TL-PVA medium supplemented with 4 μg/ml propidium iodide (PI) and 4 μg/ml bisbenzimide for 1 h. The presumptive stained blastocysts were mounted on a slide and the cells were counted under a fluorescence microscope (Olympus, Tokyo, Japan). The bisben-

zimide-stained inner cell mass (ICM) nuclei fluoresced blue, and the trophectoderm (TE) nuclei, which were stained with both bisbenzimide and PI, fluoresced red or pink.

6. Statistical Analysis

Data on the embryo development were analyzed by the χ^2-test. All cell number data were arcsine-transformed and analyzed by the General Linear Models Procedure using Statistical Analysis System software (SAS; Cary, USA). Treatment means were compared with Duncan's multiple range test; *P*-values less than 0.05 were considered statistically significant.

RESULTS

1. Sperm Storage Period

We reviewed the effectiveness of the period for which liquid sperm were stored at 17℃ on the embryo development of blastocysts or hatched blastocysts after fertilization, and the results are described in Table 1. The storage periods were 24, 48, and 72 hr.

The *in vitro* fertilization rates (≥ 2-cell) were 61.2%, 63.2%, and 58.8% for the three treatment groups. The embryo development rates to blastocysts were 9.3%, 15.3%, and 11.3% for the three groups, with no significant differences among the groups. The embryo development rates to hatched blastocysts were 11.8%, 10.3%, and 13.6% for the three groups, with no significant differences among the groups.

2. Spermatozoa Concentration

We reviewed the effect of spermatozoa concentration during *in vitro* fertilization on embryo development of blastocysts or hatched blastocysts after fertilization, and the results are described in Table 2. The spermatozoa concentrations were 0.4×10^5 cells/ml, 1.2×10^5 cells/ml, or 3.6×10^5 cells/ml.

The *in vitro* fertilization rates (≥2-cell) were 68.2%, 72.8%, and 68.9% for the 0.4×10^5 cells/ml, 1.2×10^5 cells/ml, or 3.6×10^5 cells/ml treatment groups, respectively; these values were not significantly different. The embryo development rates to blastocysts were 11.8%, 11.0%, and 11.4% for the above three groups, respectively, with no significant difference between groups. In contrast, the embryo development rates to hatched blastocysts were 8.3%, 26.5%, and 14.7%, respectively; the 1.2×10^5 cells/ml group therefore showed the highest rate, and this rate was significantly higher than that of the 0.4×10^5 cells/ml group ($p<0.05$).

3. Number of Cells

Table 3 shows the number of cells per blastocyst obtained under each condition listed in Table 2.

The total number of cells was the highest in the 1.2×10^5 cells/ml treatment group at 39.9 ± 13.7; this was significantly higher than those of the other two treatment groups (17.9 ± 5.6~25.0 ± 8.0; $p<0.05$). In contrast, the ICM cell number was 1.3 ± 0.9~2.4 ± 1.7 for all three groups, with no significant differences between groups. The TE cell number was highest in the

Table 1. The rate of fertilization and development of porcine oocytes with respect to the period of liquid sperm storage period

Liquid sperm storage period (hr)	No. of oocytes examined	No. (%) of embryo developed to		
		≥ Two-cell	Blastocysts	Hatched blastocysts
24	183	112 (61.2)	17 (9.3)	2 (11.8)
48	190	120 (63.2)	29 (15.3)	3 (10.3)
72	194	114 (58.8)	22 (11.3)	3 (13.6)

Table 2. Effect of spermatozoa concentrations during *in vitro* fertilization on the development of porcine embryos

Spermatozoa concentration (cells/ml)	No. of oocytes examined	No. (%) of embryo developed to		
		≥ Two-cell	Blastocysts	Hatched blastocysts
0.4×10^5	305	208 (68.2)	36 (11.8)	3 (8.3)[a]
1.2×10^5	309	225 (72.8)	34 (11.0)	9 (26.5)[b]
3.6×10^5	299	206 (68.9)	34 (11.4)	5 (14.7)[ab]

[a,b] Within the same columns, values with different superscripts differ significantly ($p<0.05$).

1.2×10^5cells/ml treatment group at 37.4 ± 13.5, which was significantly higher than those of the other treatment groups (16.6 ± 5.4~23.3 ± 7.6; $p<0.05$).

4. Sperm-Oocyte Co-Incubation Period

We reviewed the effect of different sperm-oocyte co-incubation periods on the embryo development to blastocysts or hatched blastocysts of *in vitro* fertilized porcine oocytes; the results are described in Table 4. The co-incubation periods were 1, 3, or 6 hours.

The *in vitro* fertilization rates were 45.1%, 60.9%, and 70.9% for the 1, 3, and 6 hr treatment groups, respectively. This values was significantly higher in the 6-hr group than in the other treatment groups ($p<0.05$). The embryo development rates to blastocysts were 2.9%, 7.5%, and 12.8% for the 1, 3, and 6 hr treatment groups, respectively and the rate was significantly higher in the 6 hr treatment group than the 1-hr group ($p<0.05$). The embryo development rates to hatched blastocysts were 0.0%, 7.7% and 27.3% for the 1, 3, and 6 hr treatment groups; these values were not significant different between groups.

5. Number of Cells

Table 5 shows the number of cells per blastocyst generated under each condition listed in Table 4.

The total number of cells was the highest in the 6-hr treatment group at 43.0 ± 7.6 cells; this value was significantly higher than those of the other treatment groups (18.6 ± 6.8~25.8 ±

Table 3. Effect of spermatozoa concentration during *in vitro* fertilization on cell numbers of porcine blastocysts developed from *in vitro*-produced embryos

Spermatozoa concentration (cells/ml)	No. of blastocysts examined	No. (%) of cells (mean ± S.E.M.)		
		ICM	TE	Total
0.4×10^5	23	1.7 ± 1.7	23.3 ± 7.6[a]	25.0 ± 8.0[a]
1.2×10^5	20	2.4 ± 1.7	37.4 ± 13.5[b]	39.9 ± 13.7[b]
3.6×10^5	20	1.3 ± 0.9	16.6 ± 5.4[a]	17.9 ± 5.6[a]

[a,b] Within the same columns, values with different superscripts differ significantly ($p<0.05$).

Table 4. Effect of spermatozoa-oocyte co-incubation periods on the development of porcine embryos

Co-incubation time (hr)	No. of oocytes examined	No. (%) of embryo developed to		
		≥ Two-cell	Blastocysts	Hatched blastocysts
1	173	78 (45.1)[a]	5 (2.9)[a]	0 (0.0)
3	174	106 (60.9)[b]	13 (7.5)[ab]	1 (7.7)
6	172	122 (70.9)[c]	22 (12.8)[b]	6 (27.3)

[a,b] Within the same columns, values with different superscripts differ significantly ($p<0.05$).

Table 5. Effect of spermatozoa-oocyte co-incubation periods on cell numbers of porcine blastocysts developed from *in vitro*-produced embryos

Co-incubation time (hr)	No. of blastocysts examined	No. (%) of cells (mean ± S.E.M.)		
		ICM	TE	Total
1	21	1.7 ± 1.9	17.0 ± 5.7[a]	18.6 ± 6.8[a]
3	20	2.0 ± 1.9	23.5 ± 5.1[a]	25.8 ± 4.2[a]
6	20	3.2 ± 1.2	39.5 ± 8.7[b]	43.0 ± 7.6[b]

[a,b] Within the same columns, values with different superscripts differ significantly ($p<0.05$).

4.2; $p<0.05$). In contrast, the ICM cell numbers were $1.7 \pm 1.9 \sim 3.2 \pm 1.2$ for the three treatment groups, with no significant differences among the groups. The highest TE cell number was detected for the 6-hr group at 39.5 ± 8.7; this value was significantly higher than those of the other treatment groups ($17.0 \pm 5.7 \sim 23.5 \pm 5.1$; $p<0.05$).

DISCUSSION

Important factors that determine the success of *in vitro* fertilization from *in vitro* matured porcine oocytes are the sperm vitality and concentration (Kikuchi *et al.*, 1993). In particular, when liquid sperm are used for *in vitro* fertilization of *in vitro* matured porcine oocytes, the bacteria in sperm are also likely to penetrate the oocyte which can cause agglutination between the sperm and a decrease in sperm motility (Althouse *et al.*, 2000). Furthermore, when sperm are exposed to antibiotics for a long time, their overall motility as well as their formation can be affected (Dahlberg, 1990). In contrast, it has been reported that sperm contamination is caused by external genitalia factors rather than internal genitalia factors (Althouse *et al.*, 2000), and contaminated sperm have different grades of contamination according to the bacterial concentration of sperm. Furthermore, differences between sperm batches can arise because of differences in storage time after sperm collection and/or degree of environmental cleaning or diluents.

Larson *et al.* (2000) reported that as the storage period of sperm increases, their motility declines, and as the bacterial number increases, functional and structural abnormalities of sperm which has a negative effect on fertilization rates and embryo development during *in vitro* fertilization. In our study, however, storage of liquid sperm for 72 hours did not adversely affect embryo development.

One of the biggest problems during *in vitro* fertilization of *in vitro* matured porcine oocytes is polyspermy. The causes of polyspermy have not yet been clearly identified; however, it is believed that this phenomenon is caused by zona pellucid consolidation after a sperm penetrates an oocytes during fertilization (Kim *et al.*, 1996; Wang *et al.*, 1997). Zona pellucid consolidation is cause by the catalytic activity of peroxidase that is secreted from the peroxisomes within the ooplasm. This phenomenon can occur in matured oocytes. However, the above phenomenon is unstable in oocytes matured in an *in vitro* culture environment, regardless of the animal species, and is more unstable for porcine species in particular. In general, greater sperm concentrations and longer sperm-oocyte co-incubation periods lead to the enhanced frequencies of polyspermic penetration (Abeydeera and Day, 1997; Marchal *et al.*, 2002; Matas *et al.*, 2003). This problem can theoretically be solved by reducing the number of sperms per ovum that are present during *in vitro* fertilization (Nagai and Moor, 1990; Abeydeera and Day, 1997). However, although a drastically reduced sperm concentration during *in vitro* fertilization can lower the polyspermic penetration rate, this also decreases the fertilization rate (Abeydeera and Day, 1997).

As described in Table 2, the embryo development rate of blastocysts after fertilization with a high concentration of sperm was similar to that when a low concentration of sperm (0.4×10^5 cells/ml) was used, but the embryo development rate of hatched blastocysts was high. An excessive spermatozoa concentration during *in vitro* fertilization adversely affects blastocyst cell number (Table 3).

Methods to increase the penetration of sperm into oocytes and to decrease the polyspermic penetration by reducing the sperm-oocyte co-incubation period during *in vitro* fertilization (Funahashi and Romar *et al.*, 2004; Gil *et al.*, 2004) (Le *et al.*, 2003) have increased the embryo development rate of blastocysts after fertilization (Alminana *et al.*, 2005). However, polyspermic penetration rates differ depending on the type of sperm used (Alminana *et al.*, 2005)., that is, fresh sperm (Funahashi and Romar, 2004) and frozen and thawed sperm (Gil *et al.*, 2004). According to the results of our study, a 6-hr sperm-oocyte co-incubation period resulted in an increase in the embryo development rate of blastocysts and the identical treatment also increased the number of blastocyst cells, consistent with the results of Koo *et al.* (2005).

To summarize, for effective *in vitro* fertilization of porcine oocytes, we recommend using liquid sperm as soon as possible. Also, a spermatozoa concentration of 1.2×10^5 cells/ml and a 6-hr sperm-oocyte co-incubation period increases the embryo development of blastocysts or hatched blastocysts after fertilization.

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