Effects of Fertilization Time and Culture Medium of Pig Oocytes Matured *In Vitro* by Liquid Boar Sperm Stored at 4℃

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체외성숙된 돼지난포란을 4℃ 보존 액상정액으로 체외수정시 수정시간과 배양배지의 영향

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ABSTRACT

This study was to investigate the effects of fertilization time and culture medium of pig oocytes matured *in-vitro* by liquid boar sperm. The sperm rich fraction (30~60 ml) was slowly cooled to room temperature (20~23℃) by 2 h after collection. Semen was transferred into 15 ml tubes, centrifuged at room temperature for 10 min 800× g, and the supernatant solution was poured off. The concentrated sperm was resuspended with 5 ml of the LEN diluent to provide 1.0×10^9 sperm/ml at room temperature. The resuspended semen was cooled in a refrigerator to 4℃. The medium used for oocyte maturation was TCM-199 supplemented with 26.19 mM sodium bicarbonate, 0.9 mM sodium pyruvate, 10 µg/ml insulin, 2 µg/ml vitamin B_{12}, 25 mM HEPES, 10 µg/ml bovine apotransferrin, 150 µM cysteamine, 10 IU/ml PMSG, 10 IU/ml hCG, 10 ng/ml EGF, 0.4% BSA, 75 µg/ml sodium penicillin G, 50 µg/ml streptomycin sulfate and 10% pFF. After about 22 h of culture, oocytes were cultured without cysteamine and hormones for 22 h at 38.5℃, 5% CO_2 in air. Oocytes were inseminated with liquid boar sperm stored at 4℃ for 2 days after collection. Oocytes were coincubated for 1, 3, 6 and 9 h in 500 µl mTBM fertilization media with 1.0×10^6 sperm/ml concentration, respectively. Thereafter, oocytes were transferred into 500 µl NCSU-23, HEPES buffered NCSU-23, PZM-3 and PZM-4 culture media, respectively, for further culture of 6, 48 and 144 h. The rates of sperm penetration and male pronuclear formation were higher in the fertilization times for 6 and 9 h than in those for 1 and 3 h. The rates of cleaved oocytes were higher in the fertilization times for 6 and 9 h (85.0 and 84.6%) than in those for 1 and 3 h (61.1 and 76.8%). The percentage of blastocyst formation from the cleaved oocytes was

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highest in the fertilization time for 6 h (33.6%) than in that for 1, 3 and 9 h (11.4, 23.0 and 29.6%). Mean cell numbers per blastocyst were 32.9, 27.6, 26.3 and 24.4 in the fertilization times for 6, 9, 3 and 1 h, respectively. The rate of blastocyst from the cleaved oocytes and the number of cells per blastocyst were higher in HEPES buffered NCSU-23 culture medium than in NCSU-23, PZM-3 and PZM-4 culture media. In conclusion, we found out that liquid boar sperm stored at 4°C could be used for *in-vitro* fertilization of pig oocytes matured *in-vitro*. Also, we recommend the coincubation time of 6 h in 500 μl TBM fertilization medium with 1×10^6 sperm/ml concentration and the HEPES buffered NCSU-23 culture medium for *in-vitro* fertilization of pig oocytes matured *in-vitro*.

(Key words : *In-vitro* fertilization, Pig oocyte, Liquid boar sperm, Culture medium)

I. INTRODUCTION

Successful *in-vitro* fertilization (IVF) could be induced effectively when both fresh boar ejaculate (Nagai et al., 1984; Hamano and Toyoda, 1986) and epididymal sperm (Nagai et al., 1984) were preincubated at a high density in a suitable medium before IVF. These reports suggested that preincubation was important for sperm capacitation. However, frozen and thawed boar ejaculated sperm were capable of IVF not only after preincubation (Nagai et al., 1988), but also without preincubation (Wang et al., 1991). With the development of rapid transportation and satisfactory preservation of liquid boar semen at about 18°C (Weitze, 1991; Johnson, 1998) or 5°C (Park et al., 1992), artificial insemination of pig has increased rapidly. But IVF of *in-vitro* matured pig oocytes using liquid boar sperm stored at 4°C was not reported.

Multiple methods have been employed to reduce polyspermic penetrations, few have been successful in maintaining high sperm penetration combined with low polyspermy (Nagai and Moor, 1990; Coy et al., 1993; Funahashi and Day, 1993; Choi et al., 1995).

Numerous studies have been conducted on culture conditions for mammalian preimplantation embryos, but to validate the normality of cultured embryos, evaluation of embryo viability is necessary *in vivo* after transfer (Bavister, 1995). Several media, such as modified Whitten medium (Beckmann and Day, 1993), North Carolina State University (NCSU)-23 medium (Petters and Wells, 1993), Beltsville embryo culture medium (BECM)-3 (Dobrinsky et al., 1996) and porcine zygote medium (PZM)-3 (Yoshioka et al., 2002), are available for the successful culture of pig embryos to the blastocyst stage.

Recently, two live offsprings from a recipient were obtained by transfer of *in vitro*-produced blastocysts (Marchal et al., 2000). However, the pregnancy rate and average litter size after transfer of cultured pig embryos is apparently low (Rath et al., 1995; Macháty et al., 1998; Brüssow et al., 2000; Day, 2000). *In vitro* development of pig embryos is delayed and results of fewer cell numbers in blastocysts compared to that with *in vivo* development (Pollard et al., 1995; Macháty et al., 1998). Thus, culture conditions for pig embryos have yet to be substantially improved.

Therefore, this study was carried out to develop a liquid boar semen diluent using for 4°C preservation and to investigate the results of IVF by liquid boar sperm, and to compare the effect of the culture media on pig oocytes matured *in-vitro*.

II. MATERIALS AND METHODS

1. Semen Collection and Liquid Semen Processing

Semen was collected from one adult Yorkshire boar twice weekly. Boar was housed at Division of Animal Science and Resources, Chungnam National University. The sperm-rich fraction (30 to 60 ml) of ejaculate was collected into an insulated vacuum bottle. The sperm-rich fractions of ejaculates with greater than 85% motile sperm and NAR acrosome were used.

Semen was slowly cooled to room temperature (20 to 23°C) by 2 h after collection. Semen was transferred into 15 ml tubes, centrifuged at room temperature for 10 min at 800×g, and supernatant solution was poured off. The concentrated sperm was resuspended with 5 ml of lactose hydrate, egg yolk and N-acetyl-D-glucosamine (LEN) diluent to provide 1.0×10^9 sperm/ml at room temperature. The resuspended semen was cooled in a refrigerator to 4°C and preserved for 5 days. The composition of liquid semen diluent used for 4°C preservation in these experiments are summarized on Table 1.

2. Oocyte Collection and *In-vitro* Maturation

Ovaries were collected from prepubertal gilts at a local slaughter house and transported to the laboratory in 0.9% NaCl solution containing 75 μg /ml potassium penicillin G, 50 μg/ml streptomycin sulfate at 30~35°C. Cumulus oocyte complexes

Table 1. Composition of liquid semen diluent using for 4°C preservation

Ingredients	LEN[a] diluent
Lactose hydrate, g	11.0
Egg yolk, ml	20.0
N-acetyl-D-glucosamine, g	0.05
Distilled water, ml[b]	100

[a] LEN: lactose-egg yolk and N-acetyl-D-glucosamine.
[b] The final volume after dissolving the ingredients is 100 ml.

(COCs) were aspirated from antral follicles (3 to 6 mm in diameter) using a 18 gauge needle fixed to a 10 ml disposable syringe. COCs were washed three times in mTLP-PVA and were washed twice with a maturation medium. Thirty to forty COCs were transferred to 500 μl of the same medium that had been covered with mineral oil in a 4-well multidish (Nunc, Roskilde, Denmark) and equilibrated at 38.5°C, 5% CO_2 in air. The medium used for oocyte maturation was TCM-199 supplemented with 26.19 mM sodium bicarbonate, 0.9 mM sodium pyruvate, 10 μg/ml insulin, 2 μg/ml vitamin B_{12}, 25 mM HEPES, 10 μg/ml bovine apotransferrin, 150 μM cysteamine, 10 IU/ml PMSG, 10 IU/ml hCG, 10 ng/ml EGF, 0.4% BSA, 75 μg/ml sodium penicillin G, 50 μg/ml streptomycin sulfate and 10% pFF. About 22 h of culture, oocytes were cultured without cysteamine and hormones for 22 h at 38.5°C, 5% CO_2 in air.

3. *In-vitro* Fertilization and Culture of Oocytes

After the completion of culture of oocytes for IVM, cumulus cells were removed with 0.1% hyaluronidase in mTLP-PVA and washed twice with mTBM fertilization medium (Abeydeera and Day, 1997). Thereafter, 30~40 oocytes were transferred into each well of a 4-well multidish containing 500 μl mTBM that had been covered with mineral oil and equilibrated at 38.5°C, 5% CO_2 in air. The dishes were kept in a CO_2 incubator until spermatozoa were added for insemination.

For IVF, 0.5 ml liquid semen preserved for 2 days was diluted with 19.5 ml Beltsville thawing solution (BTS) at 4°C. Sperm were washed twice in mTLP-PVA and then resuspended with mTBM at room temperature. Diluted sperm in mTBM was preincubated at 37°C for 10 minutes and inseminated with a final concentration of 1×10^6 sperm/ml. At 6 h after IVF, oocytes were transferred in 500 μl NCSU-23, HEPES buffered (25 mM) NCSU-23,

PZM-3 and PZM-4 culture media (Yoshioka et al., 2002), respectively, for further culture of 6, 48 and 144 h.

4. Examination of Oocytes

At 12 and 48 h after insemination, oocytes were fixed for 48 h in 25% acetic acid (v:v) in ethanol at room temperature, and stained with 1% (w:v) orcein in 45% (v:v) acetic acid to examine sperm penetration, polyspermic oocyte, male pronucleus, number of penetrated sperm in oocyte and cleaved oocytes under a phase-contrast microscope at ×400 magnification. Blastocyst at 144 h were stained with Hoechst 33342 and were counted nucleus number under fluorescent microscope (Olympus, Japan).

Unless otherwise mentioned, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MD, USA).

5. Statistical Analysis

Analysis of variance (ANOVA) were carried out using the SAS package (1996) in a completely randomized design. Duncan's multiple range test was used to compare mean values of individual treatments, when the F-value was significant (P<0.05).

Ⅲ. RESULTS

1. Effect of Fertilization Time on In-vitro Fertilization

As shown in Table 2, the percentages of sperm penetration were higher in fertilization times for 6 and 9 h than in those for 1 and 3 h. The percentage of polyspermic oocyte was highest in fertilization time for 9 h compared with other sperm concentrations. Male pronucleus formations were higher in fertilization times for 6 and 9 h than in those for 1 and 3 h. Mean number of sperm in penetrated oocyte was highest in fertilization time for 9 h.

2. Effect of Fertilization Time on Embryo Development

As shown in Table 3, the rate of blastocysts from the cleaved oocytes (2~4 cell) was highest in fertilization time for 6 h compared with other insemination times. Also, number of cells per blastocyst was highest in fertilization time for 6 h.

3. Comparison of Culture Medium in Pig Embryos

As shown in Table 4, the rate of blastocyst from the cleaved oocytes (2~4 cell stage) was highest in the HEPES buffered NCSU-23 culture medium. Number of cells per blastocyst was higher in HEPES buffered NCSU-23 culture medium than in NCSU-23, PZM-3 and PZM-4 culture media.

Ⅳ. DISCUSSION

Several liquid boar semen diluents are used throughout the world by commercial artificial insemination centers. Among them, Modena (Moretti, 1981) is one of the most widely used diluents. Summermatter (1984) modified Modena diluent by adding cysteine and BSA, and increasing the proportion of glucose, and used it as Bütschwiler. The above diluents have generally been the most successful within the storage temperature of 15 to 18°C. Chung et al. (1989) reported that liquid semen using BF_5 diluent can be stored at 5°C for 9 days in 5 ml straw. Yi et al. (2002) modified lactose-egg yolk (LEY) diluent by adding 0.05% soluble N-acetyl-D-glucosamine, and used it as LEN. The above diluent significantly enhanced the cryopreservation of boar sperm. In this study, the first diluent of the LEN without glycerol was used for liquid boar semen preservation at 4°C. We found out that the liquid boar semen could be preserved for 5 days in this diluent. It has been generally accepted that fresh ejaculated mammalian sperm acquire the

Table 2. Effect of fertilization time on fertilization parameter of pig oocytes matured *in-vitro*[1]

Fertilization time (h)	No. of oocytes inseminated	% of oocytes penetrated[2]	% of polyspermic oocytes[2]	% of oocytes with male pronucleus[2]	Mean no. of sperm in penetrated oocyte[2]
1	116	16.3 ± 4.0^c	4.2 ± 1.9^d	14.7 ± 3.9^c	0.7 ± 0.2^c
3	117	55.4 ± 3.6^b	17.4 ± 1.8^c	51.4 ± 4.8^b	1.2 ± 0.2^c
6	117	81.3 ± 1.6^a	38.4 ± 3.3^b	74.2 ± 1.6^a	3.5 ± 0.6^b
9	113	86.2 ± 3.2^a	60.3 ± 3.2^a	78.2 ± 2.0^a	5.7 ± 0.7^a

[1] HEPES buffered NCSU-23 culture medium was used in this experiment.
[2] Mean$\pm$SE. Experiments were repeated five times.
[abcd] Values in the same column with different superscripts differ significantly (P<0.05).

Table 3. Effect of fertilization time on developmental ability of pig embryos[1]

Fertilization time (h)	No. of oocytes cultured[2]	% of cleaved oocytes[3,4]	% of blastocyst from cleaved oocytes[3,4]	No. of cells per blastocyst[4]
1	93	61.1 ± 2.8^c	11.4 ± 2.4^c	24.4 ± 1.8^b
3	104	76.8 ± 2.4^b	23.0 ± 1.8^b	26.3 ± 2.2^{ab}
6	105	85.0 ± 2.3^a	33.6 ± 2.7^a	32.9 ± 3.3^a
9	105	84.6 ± 2.1^a	29.6 ± 3.1^{ab}	27.6 ± 2.7^{ab}

[1] HEPES buffered NCSU-23 culture medium was used in this experiment.
[2] Cultured oocytes were selected after *in vitro* fertilization (IVF).
[3] Rates of cleavage and blastocyst were examined at 48 and 144 h after IVF, respectively.
[4] Mean$\pm$SE. Experiments were repeated five times.
[abc] Values in the same column with different superscripts differ significantly (P<0.05).

Table 4. Effect of culture medium on developmental ability of pig embryos[1]

Culture medium	No. of oocytes cultured[2]	% of cleaved oocytes[3]	% of blastocyst from cleaved oocytes[3]	No. of cells per blastocyst
NCSU-23	149	83.9 ± 2.0^{ab}	28.2 ± 2.6^{ab}	26.1 ± 1.6^{ab}
HEPES buffered NCSU-23	145	86.2 ± 2.3^a	33.3 ± 3.0^a	28.6 ± 2.6^a
PZM-3	147	79.4 ± 1.3^{bc}	28.0 ± 2.5^{ab}	25.4 ± 2.2^{ab}
PZM-4	152	77.1 ± 1.7^c	21.0 ± 1.7^b	22.1 ± 0.7^b

[1] Mean$\pm$SE. Experiments were repeated five times.
[2] Cultured oocytes were selected after *in vitro* fertilization (IVF).
[3] Rates of cleavage and blastocyst were examined at 48 and 144 h after IVF, respectively.
[abc] Values in the same column with different superscripts differ significantly (P<0.05).

ability for penetration of ooplasm after preincubation for a certain duration at high concentration of sperm. The preincubation period of fresh ejaculated boar sperm for successful fertilization was reported to be 4~8 h (Hamano and Toyoda, 1986; Suzuki et al., 1996). When frozen-thawed ejaculated boar sperm were used for *in-vitro* fertilization (IVF), however, sperm penetration was observed without any preincubation (Wang et al., 1991).

It was suggested by Watson (1995) that the frozen-thawed sperm has already initiated capacitation. In other words, membranes of frozen-thawed sperm are more similar to those of the unfrozen-capacitated sperm, and this similarity may correspond to acrosome reaction. It was also reported that capacitated sperm increased during cooling from room temperature to 5°C and that these capacitated sperm did not need preincubation after freezing and thawing (Watson, 1996). However, the mechanism resulting capacitation without preincubation time remains unknown (Martinez, 1996).

In this study, monospermic and polyspermic sperm penetration of *in vitro* matured porcine oocytes occurred as early as 1 h postinsemination. Polyspermic fertilization continuously increased from 1 to 9 h after fertilization. The increase in penetration rate between 6 and 9 h was due to an increase in polyspermic fertilization, whereas monospermy, which was maximum after 6 h of IVF. It would be interesting to be monospermic after 9 h. It could indicate that some oocytes possess highly efficient mechanisms to prevent polyspermic penetration. An improvement of the block to polyspermy by the adjustment of *in vitro* culture conditions should then be considered (Marchal et al., 2002).

Yoshioka et al. (2002) reported that the proportion of Day 6 blastocysts and Day 8 hatching frequency were greater in embryos with PZM-3 than in those cultured with NCSU-23. However, our results showed that the HEPES buffered NCSU-23

medium has been the most successful for the culture of pig embryos compared to the NCSU-23, PZM-3 and PZM-4 media.

In this study, we found out that liquid boar sperm stored at 4°C in the lactose-egg yolk diluent with N-acetyl-D-glucosamine could be used for in-vitro fertilization of pig oocytes matured *in-vitro*.

In conclusion, fertilization time by liquid boar sperm stored at 4°C for 2 days after collection affected the rates of sperm penetration, polyspermy, male pronuclear formation of oocytes and the mean number of sperm in the penetrated oocyte, and the rate of blastocysts from the cleaved oocytes. Also, we recommend the HEPES buffered NCSU medium for porcine embryo culture.

V. 요 약

본 연구는 체외성숙된 돼지난포란을 액상정액으로 수정시 수정시간과 배양배지가 난포란의 발달에 미치는 영향을 조사하기 위하여 실시하였다. 정자농후정액 (30~60 ml)을 채취하여 실온에서 2시간 정도 서서히 냉각시킨 후, 정액을 15 ml 튜브에 담아 800×g로 10분간 원심분리하였다. 상층액은 버리고 하부의 정자는 5 ml LEN 희석액으로 1×10^9 정자/ml가 되도록 재희석하였다. 희석된 정액은 4°C 냉장고에 보존하였다. 미성숙 난모세포의 성숙에 사용된 배지는 26.19 mM sodium bicarbonate, 0.9 mM sodium pyruvate, 10 μg/ml insulin, 2 μg/ml vitamin B_{12}, 25 mM HEPES, 10 μg/ml bovine apotransferrin, 150 μM cysteamine, 10 IU/ml PMSG, 10 IU/ml hCG, 10 ng/ml EGF, 0.4% BSA, 75 μg/ml sodium penicillin G, 50 μg/ml streptomycin sulfate 그리고 10% pFF를 첨가한 TCM-199 배지였다. 22시간 성숙 배양한 후 난모세포는 cysteamine과 hormone들을 배제한 후 38.5°C, 5% CO_2 incubator에서 22시간 더 성숙시켰다. 성숙된 난모세포는 채취 후 2일간 4°C에 보존된 액상정액으로 수정되었다. 난모세포는 500 μl mTBM 수정배지에서 1×10^6 정자/ml의 농도로 1, 3, 6 그리고

9시간 동안 수정시켰다. 그 후 난모세포는 500 ㎕ NCSU-23, Hepes buffered NCSU-23, PZM-3 그리고 PZM-4 배양배지에 옮겨서 6, 48 그리고 144시간을 더 배양하였다. 정자침투율, 웅성전핵형성율 그리고 난모세포의 난할율은 6 및 9시간 수정시간에서 1 및 3시간 수정시간 보다 높았다. 6시간 수정시 배반포형성율 (33.6%)은 1, 3 그리고 9시간 수정시 배반포형성율 (11.4, 23.0 그리고 29.6%) 보다 높았다. 배반포의 평균세포수는 6, 9, 3 그리고 1시간 수정시 각각 32.9, 27.6, 26.3 그리고 24.4개였다. 분할된 난모세포의 배반포형성율 그리고 배반포의 평균세포수는 NCSU-23, PZM-3 그리고 PZM-4 배양배지보다 HEPES buffered NCSU-23 배양배지가 우수하였다. 결론적으로 4℃ 보존 돼지액상정액은 체외성숙된 돼지 난모세포의 체외수정에 사용될 수 있음이 입증되었다. 또한 체외성숙된 돼지 난모세포는 500 ㎕ mTBM 수정배지에서 1×10^6 정자/ml로 6시간 공배양시키는 것이 바람직하며, HEPES buffered NCSU-23 배양배지에서 배양하는 것이 좋다는 결과를 얻었다.

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