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Effect of Oocyte Maturation Medium, Cytochalasin Treatment and Electric Activation on Embryonic Development after Intracytoplasmic Sperm Injection in Pigs

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ABSTRACT

The objective of this study was to examine the effect of in vitro maturation (IVM) medium, cytochalasin B (CB) treatment during intracytoplasmic sperm injection (ICSI), and electric activation on *in vitro* development ICSI-derived embryos in pigs. Immature pig oocytes were matured *in vitro* in medium 199 (M199) or porcine zygote medium (PZM)-3 that were supplemented with porcine follicular fluid, cysteine, pyruvate, EGF, insulin, and hormones for the first 22 h and then further cultured in hormone-free medium for an additional 21～22 h. ICSI embryos were produced by injecting single sperm directly into the cytoplasm of IVM oocytes. The oocytes matured in PZM-3 with 61.6 mM NaCl (low-NaCl PZM-3) tended to decrease ($0.05<P<0.1$) nuclear maturation when compared with oocytes matured in M199 (76.9% vs. 83.8%) but no significant differences were found in embryo cleavage, blastocyst formation, and mean number of cells in blastocyst (73.8% vs. 74.6%, 11.1% vs. 12.1%, and 28.4 cells vs. 30.1 cells, respectively). The oocyte degeneration was not reduced by CB treatment during ICSI (11.9%) when compared with no treatment control (11.3%) while the treatment showed detrimental effects ($P<0.05$) on embryonic cleavage (40.0%) and blastocyst formation (1.8%) rates when compared with control (60.0% and 11.5%, respectively). For activation of ICSI oocytes, additional electric stimulus has no positive or negative effect on *in vitro* development of preimplantation stage ICSI porcine embryos. Our results demonstrate that CB treatment during ICSI inhibits embryonic development of ICSI oocytes and additional electric activation after ICSI has no effect in improving ICSI embryonic development in pigs. Further studies are needed to improve ICSI efficiency by investigating factors influencing embryonic development after ICSI in pigs.

(Key words : cytochalasin B, electric activation, embryonic development, ICSI, pig)

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) is a useful technique and can be used to maximize the utilization of male genetic resources including spermatozoa and round spermatids. This technique was developed in hamsters in the 1970s (Uehara and Yanagimachi, 1976, 1977) and has been used subsequently to achieve fertilization and live births in rabbits, cattle, and humans (Iritani *et al*., 1988; Lanzendorf *et al*., 1988; Goto *et al*., 1990; Palermo *et al*., 1992). Presently, ICSI is an important tool for investigating and understanding the early events of fertilization, and is used as an assisted reproductive technology (ART) in humans. For decades, a variety of studies has been performed for the efficient production of mammalian embryos by using ARTs like ICSI and *in vitro* fertilization (IVF). It is well known that embryos derived from *in vitro* maturation (IVM), IVF and ICSI show lower developmental capacity than embryos derived *in vivo*. Even though many species have been successfully generated as live ICSI-derived offspring, the efficiency of live ICSI-derived piglets production

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has only been reported as 4～6% in pigs (Katayama *et al*., 2007; Watanabe *et al*., 2012), which means the current pig ICSI-*in vitro* production (IVP) system is suboptimal and needs to be improved.

Developmental competence of IVP pig embryos is influenced by various factors including oocytes maturation, culture media, and other culture environments (Park *et al*., 2005; Viet Linh *et al*., 2009; Kim *et al*., 2010; Lee *et al*., 2013). Of those factors, the quality of oocytes may be one of the most critical factors in determining the success of IVP of pig embryos by IVF, ICSI, and somatic cell nuclear transfer (SCNT) (Yoon *et al*., 2000; Kim *et al*., 2010). Recently, it was found that lowering NaCl concentration in IVM medium could improve the cytoplasmic maturation and subsequent development of embryos derived from parthenogenetic activation and SCNT (Lee *et al*., 2013) but no report is available on the effect of IVM medium with low concentration of NaCl on the ICSI embryonic development.

In nuclear transfer manipulation, cytochalasin B (CB) relaxes the cytoskeleton and enhances the flexibility of the oocyte. Treating oocytes of various species with CB during nuclear transfer prevented damage to the oolemma, allowed the insertion of a glass pipette through the ZP without lysing the oocyte (Baguisi *et al*., 1999; Polejaeva *et al*., 2000), and resulted in an improved success rate of embryonic development during the cloning of rabbits and sheep (Smith and Wilmut, 1989; Collas and Robl, 1990).

ICSI also needs mechanical manipulation of oocytes to inject sperm into the oocyte cytoplasm. Presently, studies describing the effects of CB-supplemented manipulation media on oocyte and embryonic development following ICSI are rarely reported. Spermatozoa derived from several mammalian species are also unable to activate oocytes, mainly due to lack of oocyte activation factors. Recent studies have shown that species differences exist in oocyte activation after ICSI. An additional activation procedure is required to allow oocyte fertilization following spermatozoa injection in cattle (Rho *et al*., 1998) and pigs (Lee *et al*., 2003), whereas injection alone is sufficient to activate oocytes in mice and humans (Tesarik, 1996). Although mechanical stimulation by the injection pipette occasionally results in activation, further stimulation may be needed for porcine oocytes.

The objective of this study was to examine the effect of IVM medium (M199 and low-NaCl PZM-3), CB treatment during ICSI, and additional electric activation after ICSI on *in vitro* development ICSI-derived embryos in pigs.

MATERIALS AND METHODS

1. Culture Media

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, USA). The base medium for IVM of oocytes was medium199 (M199) (Invitrogen, Grand Island, USA) or low-NaCl PZM-3 (61.6 mM) supplemented with 0.6 mM cysteine, 0.91 mM pyruvate, 10 ng/ml epidermal growth factor, 75 μg/ml kanamycin, 1 μg/ml insulin, and 10 % (v/v) porcine follicular fluid. Two media, PZM-3 (Yoshioka *et al*., 2002) and mPZM-3 (Lee *et al*., 2012) were used for *in vitro* culture (IVC) of ICSI embryos. PZM-3 was modified by supplementation with 2.77 mM myo-inositol, 0.34 mM trisodium citrate, and 10 μM β-mercaltoehtanol (this medium was designated mPZM-3) according to the experimental design.

2. Oocyte Collection and IVM

Porcine ovaries were obtained from prepubertal gilts at a local abattoir and transported to the laboratory. Cumulus-oocyte complexes (COCs) were aspirated from superficial follicles (3～8 mm in diameter) in the ovaries using an 18-gauge needle and a 10 ml syringe. COCs that had multiple layers of compacted cumulus cells were selected and washed three times in HEPES-buffered Tyrode's medium containing 0.05% (w/v) polyvinyl alcohol (TLH-PVA). COCs were placed into each well of a four-well multi-dish (Nunc, Roskilde, Denmark) containing 500 μl of IVM medium with 80 μg/ml FSH (Antrin R-10, Kyoritsu Seiyaku, Japan) and 10 IU/ml hCG (Intervet International BV, Holland). The COCs were cultured at 39℃ with 5% CO_2 at maximum humidity. After 22 h in the maturation culture, the COCs were washed three times in fresh hormone-free IVM medium and then cultured in hormone-free IVM medium for an additional 21～22 h for ICSI.

3. ICSI

After IVM, COCs with expanded cumulus cells were treated with 0.1 % (w/v) hyaluronidase in IVM medium, and the cumulus cells were removed by repeated gentle pipetting. Denuded oocytes were placed in droplets of TLH containing 0.4 % BSA, and oocytes with a uniform cytoplasm were chosen under the microscope. Spermatozoa for ICSI were prepared from extended liquid boar semen commercially available. Spermatozoa were washed two times with D-PBS containing 0.1%

PVA and centrifuged at 700 g for 3 min. The sperm pellet was resuspended in D-PBS containing 5% polyvinyl pyrrolidone (PVP). Microinjection of whole spermatozoa into oocytes was performed at 39℃ using a 5.0~5.7 μm pipette (Origio Humagen, Charlottesville, USA). A sperm was aspirated into the injection pipette with a minimal amount of medium and introduced into the cytoplasm. ICSI was performed in the presence or absence of 5 μg/ml CB in manipulation medium. ICSI was completed within 2 h after collection of IVM oocytes.

4. Electric Activation of ICSI Oocytes and Embryo Culture

All oocytes used for ICSI were washed three times with IVC medium. After washing, ICSI embryos were activated with two pulses of 120 V/mm DC for 60 μsec in a 280 mM mannitol solution containing 0.01 mM $CaCl_2$ and 0.05 mM $MgCl_2$ according to the experimental design. Activated or non-activated ICSI embryos were washed three times in fresh culture medium, transferred into 30-μl droplets of IVC medium under mineral oil, and then cultured at 39℃ in a humidified atmosphere of 5 % CO_2, 5% O_2, and 90% N_2 for 7 days.

5. Experimental Design

In Experiment 1, the effects of two maturation media, M199 and low-NaCl PZM-3 on nuclear maturation and embryonic development of pig oocytes after ICSI were compared. It was examined in Experiment 2 whether CB supplementation to the manipulation medium during ICSI would prevent oocyte degeneration and improve embryonic development of ICSI oocytes. In addition, effect of additional activation of ICSI oocytes by electric stimulus on embryonic development was examined in Experiment 3. Degeneration of ICSI oocytes was examined 2 h after sperm injection. Embryo cleavage and blastocyst formation were evaluated on Days 2 and 7, respectively (the day of ICSI was designated as Day 0). The mean cell number in blastocysts developed on Day 7 was examined using Hoechst 33342 staining using an epifluorescence microscope.

6. Statistical Analysis

All statistical analyses were performed using the Statistical Analysis System (version 9.3; SAS Institute, USA). Data were analyzed using a general linear model followed by the least square method when the treatments differed at $P<0.05$. Percentage data were arcsine-transformed prior to analysis to maintain the homogeneity of variances. The results are expressed as the mean ± standard error of the mean (SEM).

RESULTS

1. Experiment 1: Preimplantation Development of ICSI Emb- ryos that were Matured in Two Different Culture Media: M199 and low-NaCl PZM-3

Maturation culture of oocytes in PZM-3 with 61.6 mM NaCl (low-NaCl PZM-3) tended to decrease ($0.05<P<0.1$) nuclear maturation compared to maturation in M199 (76.9% vs. 83.8%). No significant differences were found in embryo cleavage, blastocyst formation, and mean number of cells in blastocyst (73.8% vs. 74.6%, 11.1% vs. 12.1%, and 28.4 cells vs. 30.1 cells, respectively) (Table 1).

2. Experiment 2: Effect of CB Treatment during ICSI on Oocyte Degeneration and Subsequent Embryonic Development

CB treatment during ICSI did not reduce oocyte degeneration (11.9%) compared to no treatment (11.3%). Instead, CB significantly reduced embryo cleavage (40.0% vs. 60.0%) and blastocyst formation (1.8% vs. 11.5%) compared to control (Table 2).

3. Experiment 3: Effect of Additional Activation of ICSI Oocytes by Electric Stimulus on Preimplantation Development of ICSI Embryos

Activation of ICSI oocytes by electric stimulus did not alter embryonic development to the cleavage (78.6% vs. 76.1%) and

Table 1. *In vitro* development of intracytoplasmic sperm injection (ICSI)-derived pig embryos that were matured *in vitro* in medium 199 and PZM-3 with 61.6 mM NaCl (Low-NaCl PZM-3)

In vitro maturation medium	% of oocytes reached metaphase II	No. of ICSI embryos cultured	% of embryos developed to*		No. of cells in blastocyst
			≥ 2-cell	Blastocyst	
Medium 199	83.8 ± 3	135	74.6 ± 8	12.1 ± 3	30.1 ± 4
Low-NaC lPZM-3	76.9 ± 3	137	73.8 ± 7	11.1 ± 3	28.4 ± 2

Six replicates.

* Percentage of the number of ICSI embryos cultured.

Table 2. Effect of cytochalasin B (CB) treatment during intracytoplasmic sperm injection (ICSI) on degeneration of oocytes and subsequent embryonic development after ICSI

CB treatment during ICSI	% of oocytes degenerated after ICSI	No. of ICSI embryos cultured	% of embryos developed to*		No. of cells in blastocyst
			≥ 2-cell	Blastocyst	
No (control)	11.3 ± 4	112	60.0 ± 7[a]	11.5 ± 2[a]	27.2 ± 4
Yes	11.9 ± 3	112	40.0 ± 4[b]	1.8 ± 1[b]	24.0 ± 6

Six replicates.
* Percentage of the number of ICSI embryos cultured.
[a,b] Different superscript letters indicates a significant difference within a column (P<0.05).

Table 3. Effect of electric activation on embryonic development of pig oocytes after ICSI

Electric activation	% of oocytes degenerated after ICSI	No. of ICSI embryos cultured	% of embryos developed to*		No. of cells in blastocyst
			≥ 2-cell	Blastocyst	
No (control)	3.3 ± 2	58	76.1 ± 7	8.6 ± 2	29.4 ± 4
Yes	5.0 ± 5	57	78.6 ± 6	5.5 ± 1	25.7 ± 4

Three replicates.
* Percentage of the number of ICSI embryos cultured.

the blastocyst stages (5.5% vs. 8.6%) compared to control (Table 3).

DISCUSSION

Developmental competence of IVP embryos by IVF, ICSI, or SCNT is influenced by various factors. In this study, we examined the effects of several factors such as oocyte maturation medium, CB treatment during ICSI, and electrical activation of ICSI oocytes on subsequent embryonic development. It was demonstrated that CB treatment during ICSI inhibits embryonic development of ICSI oocytes while maturation of oocytes in low-NaCl PZM-3 and additional electric activation after ICSI has no effect in improving ICSI embryonic development in pigs.

The quality of oocytes is one of the most important factors determining the success of ART in various species (Wang *et al*., 1998). Recently, it has been reported that oocytes having a wide perivitelline space show lower incidence of polyspermic fertilization after IVF (Funahashi *et al*., 1994; Wang *et al*., 1998) and higher developmental competence after parthenogenetic activation (PA) and somatic cell nuclear transfer (SCNT) (Lee *et al*., 2013). In this study, embryonic development of ICSI oocytes was not improved by the maturation of oocytes in low-NaCl PZM-3. This result was not consistent with the previous result that embryonic development of PA and SCNT embryos was increased by the culture of oocytes in low-NaCl PZM-3 (Lee *et al*., 2013). The rate of embryos developed to the blastocyst state was around 10% that was lower than that of PA or SCNT embryos in our previous study. It was not clear the reason why no improving effect was shown in ICSI embryonic development. Relatively low developmental competence of ICSI oocytes might be too low to be improved by the beneficial effect of IVM in low-NaCl PZM-3.

ICSI is a manipulative technique in which a single sperm is injected directly into the ooplasm of a mature oocyte using a micropipette. When sperm is injected into the oocyte cytoplasm, oocytes can be ruptured or degenerated by mechanical damage to the ooplasmic membrane or cytoplasmic structures. During the manipulation of oocytes for the enucleation or donor cell injection in SCNT, CB is commonly used to prevent oocytes from degenerating by stabilizing oocyte cytoskeletons (Sugimura *et al*., 2008; Song *et al*., 2009). Treatment of oocytes with CB during sperm injection had no effect in preventing oocyte degeneration and was detrimental to embryonic development of ICSI oocytes. It was considered that diameter of ICSI pipette was too small to result in mechanical damage to the oocytes. CB inhibits cytoplasmic division by

blocking the formation of contractile microfilaments. Although nuclear remodeling of ICSI oocytes was not examined in this study, it was probable that CB treatment during sperm injection affected nuclear remodeling such as inhibition of polar body extrusion in ICSI oocytes and resultantly reduced embryonic development. This result indicates that exposure of ICSI oocytes to CB for short time (within 15 min) is detrimental to embryonic development.

In the normal fertilization process, sperm has to be capacitated and acrosome reaction is necessary before penetration (Matas *et al*., 2010; Tsai *et al*., 2010). Once sperm has penetrated into the oocytes, oocytes are activated by the signal from the penetrated sperm (Wang *et al*., 1998). In ICSI, sperm is directly injected into oocytes without either acrosome reaction or capacitation processes. This is not physiological process and thus it is suspected whether the same pattern of oocyte activation will occur in ICSI oocytes as in normal fertilized oocytes. Electric activation has been widely used for activation of ICSI or SCNT oocytes (Prather *et al*., 1985; Jolliff *et al*., 1997; Wang *et al*., 1998; Kure-bayashi *et al*., 2000). Oocytes activation by electric stimulus in this study showed neither beneficial nor detrimental effects on embryonic development of ICSI oocytes. Our result was not consistent with previous reports in which electric activation of ICSI oocytes increased embryo cleavage and blastocyst formation (Lee *et al*., 2003; Yoo *et al*., 2011) but was similar with the result that electrical activation was not necessary in the modified porcine ICSI (Yong *et al*., 2005). From present and previous results, it is considered that the effect of electric activation on ICSI embryonic development can be various depending on the method of sperm injection and culture environments of ICSI oocytes. In summary, our results demonstrate that CB treatment during ICSI inhibits embryonic development of ICSI oocytes and additional electric activation after ICSI has no effect in improving ICSI embryonic development in pigs. Further studies are needed to improve ICSI efficiency by investigating factors influencing embryonic development after ICSI in pigs.

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