A Comparative Study on the Parthenogenetic Development of Pig Oocytes Cultured in North Carolina State University-23 and Porcine Zygote Medium-3

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ABSTRACT

The objective of this study was to examine the effect of *in vitro* culture media on embryonic development of *in vitro*-matured (IVM) oocytes after parthenogenetic activation (PA) in pigs. Immature pig oocytes were matured in TCM-199 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 22～26 h. IVM oocytes were activated by electric pulses and cultured in porcine zygote medium-3 (PZM-3) and North Carolina State University-23 supplemented with essential and non-essential amino acids (NCSU-23aa). These media were further modified by supplementing 2.77 mM myo-inositol, 0.34 mM trisodium citrate, and 10 μM β-mercaptoethanol (designated as mPZM-3 and mNCSU-23aa, respectively). Culture of PA embryos in mPZM-3 significantly increased development to the blastocyst stage than culture in NCSU-23aa (36.2% vs. 24.8%, $p<0.05$). Modified PZM-3 showed a significantly higher blastocyst formation than NCSU-23aa in both groups of embryos that were activated at 44 h and 48 h of IVM (51.0% vs. 35.5% and 49.0% vs. 34.2% in oocytes activated at 44 h and 48 h of IVM, respectively). Irrespective of the follicle diameter where oocytes were collected, embryonic development to the blastocyst stage was increased ($p<0.05$) by the culture in mPZM-3 compared to culture in NCSU-23aa (25.9% vs. 34.2% and 32.9% vs. 44.8% in embryos derived from small and medium size follicles, respectively). Our results demonstrated that culture media had significant effect on preimplantation development PA embryos and that mPZM-3 was superior to mNCSU-23 in supporting development to the blastocyst stage in pigs. This beneficial effect of mPZM-3 on embryonic development was not impaired by other factors such as time of oocyte activation and origin of immature oocytes (small and medium size follicles).

(Key words : parthenogenesis, embryonic development, NCSU-23, PZM-3, pig)

INTRODUCTION

In vitro production techniques using *in vitro* maturation (IVM) of follicular oocytes and culture of embryos after *in vitro* fertilization (IVF), somatic cell nucleus transfer (SCNT), and intracytoplasmic sperm injection have been used for production of genetically superior animals and transgenic animals having specific genes (Wilmut *et al.*, 1997; Polejaeva *et al.*, 2000; Staunstrup *et al.,* 2012). In addition, embryos produced by parthenogenetic activation (PA) of oocytes can be used to establish an embryonic stem cell lines or as a model to test effect of culture media on *in vitro* development of IVF and SCNT embryos (You *et al.,* 2010; Isom *et al.,* 2012). For decades, a variety of study on the efficient production of mammalian embryos using assisted reproductive technology has been performed by many workers. It has been reported that developmental competence of 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). It has been known that embryos derived from IVM and IVF show lower developmental capacity than embryos derived *in vivo*, which means the current pig IVP system is suboptimal and needs to be improved.

A culture medium is one of the critical factors influencing embryonic development *in vitro* (Park *et al.,* 2005; Wu *et al.,* 2011). Several culture media that were designed for each species such as synthetic oviductal fluid in bovine (Tervit *et al.,* 1972; Fukui *et al.,* 1991) and porcine zygote medium-3 (PZM-

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3) in porcine have been used to produce embryos *in vitro*. In pigs, North Carolina State University Medium (NCSU)-23 without (Petters and Wells, 1993) or with essential and non-essential amino acids (designated as NCSU-23aa) has been widely used for pig embryo culture (Hashem *et al.,* 2006; Yamanaka *et al.,* 2009), but a new medium, PZM-3, was designed based on the composition of pig oviductal fluid. The main difference from NCUS- 23 was composition of energy substrate and potassium ion concentration (Yoshioka *et al.,* 2002). Glucose is not included and the concentration of potassium ion is high in PZM-3 compared to NCSU-23. These two media were compared for the effect on pig embryonic development by several workers but there was limited information on the development of PA embryos that were produced by various methods such as applying different time of activation and different source of oocytes from small and medium size follicles. In addition, no study is available that examined supplementing effect with myo-inositol, trisodium citrate, and β-mercaptoethanol (β-ME) of PZM-3 and NCSU-23 (designated as mPZM-3 and mNCSU-23aa, respectively).

The objective of this study was to examine and compare the effect of *in vitro* culture media (mPZM-3, NCSU-23aa, and mNCSU-23aa) on embryonic development of PA oocytes in pigs. It was demonstrated in this study that culture media for pig embryos showed significant effect on preimplantation development PA embryos. Modified PZM-3 was superior to mNCSU-23aa in supporting *in vitro* development of PA embryos to the blastocyst stage. This beneficial effect of mPZM-3 on embryonic development was not altered by other factors such as time of oocyte activation and origin of immature oocytes (small and medium size follicles).

MATERIALS AND METHODS

1. Culture Media

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The base medium for IVM of oocytes was medium-199 (M-199) (Invitrogen, Grand Island, NY, USA) 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, NCSU-23aa (Petters and Wells, 1993; Park *et al.,* 2005) and PZM-3 (Yoshioka *et al.,* 2002) containing essential and non-essential amino acids were used for *in vitro* culture (IVC) of PA embryos (Table 1). These media were modified by supplementation with 2.77 mM myo-inositol, 0.34 mM trisodium citrate, and 10 μM β-ME according to the experimental design.

2. Oocyte Collection and IVM

Ovaries were obtained from prepubertal gilts at a local abattoir. Follicular contents were aspirated from the superficial follicles of the ovaries with an 18-G needle attached to a 10-mL disposable syringe. Oocytes were separately aspirated from small (< 3 mm in diameter) and medium size (3~8 mm in diameter) follicles and cultured for IVM and parthenogenetic development according to the experimental design. Cumulus-oocyte-complexes (COCs) with more than three layers of com-

Table 1. Composition of *in vitro* culture media used in this study

Component	Culture medium			
	PZM-3	mPZM-3	NCSU-23aa	mNCSU-23aa
NaCl (mM)	108.00	108.00	108.73	108.73
KCl (mM)	10.00	10.00	4.78	4.78
$CaCl_2 \cdot 2H_2O$ (mM)	-	-	1.70	1.70
KH_2PO_4 (mM)	0.35	0.35	1.19	1.19
$NaHCO_3$ (mM)	25.07	25.07	25.07	25.07
Glucose (mM)	-	-	-	-
Sodium pyruvate (mM)	0.2	0.2	0.5	0.5
Sodium lactate (mM)	-	-	5.0	5.0
Ca-(lactate)$_2 \cdot 5H_2O$ (mM)	2.0	2.0	-	-
L-Glutamine (mM)	1.0	1.0	1.0	1.0
BME amino acids (%) (v/v)*	2	2	2	2
MEM NEAA (%) (v/v)**	1	1	1	1
Taurine (mM)	-	-	7	7
Hypotaurine (mM)	5	5	5	5
Fatty acid-free BSA (mg/ml)	3	3	4	4
Myo-inositol (mM)	-	2.77	-	2.77
Trisodium citrate (mM)	-	0.34	-	0.34
β-Mercaptoethanol (μM)	-	10	-	10
pH	7.3	7.3	7.3	7.3

* BME amino acids; Basal Medium Eagle amino acids.
** MEM NEAA; Minimal Essential Medium non-essential amino acids.

pact cumulus cells were selected, washed three times in a HEPES-buffered Tyrode's medium containing 0.05% (w/v) PVA (TLH-PVA) (Bavister *et al.*, 1983), and then washed once in IVM medium. Groups of 40-70 COCs from each category were placed into individual wells of a 4-well multi-dish (Nunc, Roskilde, Denmark) that contained 500 μl IVM medium supplemented with 10 IU/ml equine chorionic gonadotropin (Intervet International BV, Boxmeer, Holland) and 10 IU/ml human chorionic gonadotropin (Intervet) and cultured at 39℃ in a humidified atmosphere of 5% CO_2. After 22 h in the maturation culture, the COCs were washed three times in fresh, hormone-free IVM medium and cultured for an additional 22～26 h according to the experimental design.

3. PA, Post-Activation Treatment, and Embryo Culture

After 44 or 48 h of IVM, oocytes were denuded and cumulus-cell-free oocytes were examined for maturation. Oocytes having the first polar body were considered as matured. Mature oocytes 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 $MgCl_2$. Following electrical activation, the PA embryos were treated with 5 μg/ml cytochalasin B in IVC medium for 4 h (Song *et al.,* 2009). The PA embryos were washed three times in fresh IVC 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. Cleavage and blastocyst formation were evaluated on Days 2 and 7, respectively (the day of PA designated Day 0). The mean cell number in blastocysts developed on Day 7 was examined using Hoechst 33342 staining under an epifluorescence microscope.

4. Experimental Design

In Experiment 1, effect of IVC medium on the embryonic development of pig IVM oocytes after PA was examined. Oocytes collected from medium size follicle were used for production of PA embryos in Experiments 1 and 2. Based on the result from Experiment 1, it was examined in Experiment 2 whether the mPZM-3 could improve the developmental competence of PA embryos that were activated at different time of IVM (44 and 48 h of IVM). Finally, it was examined whether the beneficial effect of mPZM-3 was attributed to the supplementation with myo-inositol, trisodium citrate, and β-ME and would be shown even in the development of PA embryos that were derived from small size follicles in Experiment 3.

5. Statistical Analysis

All statistical analyses were performed using the Statistical Analysis System (version 9.1; SAS Institute, Cary, NC, 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.

RESULTS

Experiment 1: Preimplantation Development of Parthenogenetic Embryos Cultured in Two Different Culture Media: NCSU-23aa and Modified PZM-3

Effect of IVC medium on the embryonic development of pig IVM oocytes after PA is shown in Table 2. Culture of PA embryos in mPZM-3 showed an increased development to the blastocyst stage than culture in NCSU-23aa (36.2% vs. 24.8%, $p<0.05$). Moreover, mean cell number of embryos was higher in blastocysts that were developed from the culture in mPZM-3 compared to NCSU-23aa (31.7 cells vs. 36.5 cells, $p<0.05$).

Experiment 2: Effect of IVC Medium on the Preimplantation Development of Parthenogenetic Embryos activated at 44 h and 48 h of IVM

Modified PZM-3 significantly ($p<0.05$) increased blastocyst formation of PA embryos compared to NCSU-23aa irrespective of the time of oocyte activation for PA (Table 3). In oocytes that were activated at 44 h of IVM, a significant difference in blastocyst formation of PA embryos was found. The rates of oocytes that formed blastocyst were 51.0% and 35.5% in mPZM-3 and NCSU-23aa, respectively. When oocytes were activated at 48 h of IVM, the rates blastocyst formation of PA embryos that were cultured in mPZM-3 and NCSU-23aa were 49.0% and 34.2%, respectively.

Experiment 3: Effect of IVC Medium on the Preimplantation Development of Parthenogenetic Embryos Derived from Small and Medium Size Follicles

Irrespective of the follicle size where oocytes were derived, embryonic development to the blastocyst stage was increased ($p<0.05$) by the culture in mPZM-3 compared to culture in NCSU-23aa (25.9% vs. 34.2% and 32.9% vs. 44.8% in embryos derived from small and medium size follicles, respectively). NCSU-23 that was modified by the supplementation with myo-inositol, trisodium citrate, and β-ME (mNCSU-23) showed a

Table 2. Effect of *in vitro* culture medium on the development of parthenogenetic pig oocytes matured *in vitro*

In vitro culture medium	No. of embryos cultured	% of embryos developed to		No. of cells in blastocyst
		≥ 2-cell	Blastocyst	
NCSU-23aa	440	86.8	24.8[a]	31.7[a]
mPZM-3	458	88.9	36.2[b]	36.5[b]

[a,b] Different superscript letters indicates a significant difference within a column ($p<0.05$).

Table 3. Effect of *in vitro* culture medium on the development of parthenogenetic pig oocytes activated at 44 and 48 h of *in vitro* maturation

In vitro culture medium	Time of activation after IVM	No. of embryos cultured	% of embryos developed to		No. of cells in blastocyst
			≥ 2-cell	Blastocyst	
44 h	NCSU-23aa	155	95.5	35.5[a]	31.5
	mPZM-3	155	93.5	51.0[b]	31.5
48 h	NCSU-23aa	155	92.9	34.2[a]	30.4
	mPZM-3	151	92.7	49.0[b]	32.5

[a,b] Different superscript letters indicates a significant difference within a column ($p<0.05$).

similar rate of embryos that developed to the blastocyst stage to mPZM-3 (31.6% vs. 34.2% and 36.9% and 44.8% in embryos derived from small and medium size follicles, respectively) (Table 4).

DISCUSSION

Various culture media such as Whitten's medium, TCM-199, NCUS-23, and PZM-3 have been used for IVC of pig embryos. It has been shown that pig embryonic development to the blastocyst stage is significantly influenced by the culture medium (Dobrinsky *et al.,* 1996; Yoshioka *et al.,* 2002; Park *et al.,* 2005). A culture medium consists of various inorganic components such as NaCl, $CaCl_2$, $MgSO_4$, and KCl, amino acids, energy substrates such as glucose, pyruvate, and lactate. In this study, two culture media, NCSU-23aa and mPZM-3 that was designed based on the composition of pig oviductal fluid, were compared for their effect on embryonic development of pig oocytes after PA. Our results confirmed again the previous results (Yoshioka *et al.,* 2002; Im *et al.,* 2004) that PZM-3 was superior to NCSU-23aa in supporting embryonic development and that culture medium was a very important factor in-

Table 4. *In vitro* development of parthenogenetic pig oocytes with different size after *in vitro* maturation cultured three different culture media

Follicle size for oocyte retrieval	*In vitro* culture medium	No. of embryos cultured	% of embryos developed to		No. of cells in blastocyst
			≥ 2-cell	Blastocyst	
Small (<3 mm in diameter)	NCSU-23aa	320	84.4	25.9[a]	29.3[a]
	mNCSU-23aa	320	87.2	31.6[ab]	33.4[ab]
	mPZM-3	325	89.8	34.2[b]	37.8[b]
Medium (3~8 mm in diameter)	NCSU-23aa	386	91.2	32.9[ab]	31.5[ab]
	mNCSU-23aa	385	88.3	36.9[b]	30.9[ab]
	mPZM-3	386	92.2	44.8[bc]	35.6[b]

[a~c] Different superscript letters indicates a significant difference within a column ($p<0.05$).

fluencing preimplantation development of mammalian embryos.

In this study, mPZM-3 significantly increased embryonic development to the blastocyst stage of PA pig oocytes compared to NCSU-23aa. This result was consistent with the previous result (Yoshioka *et al.,* 2002) that PZM-3 was more effective than NCSU-23 in supporting preimplantation development of *in vivo*-fertilized pig embryos. This stimulating effect of PZM-3 was also shown in preimplantation development of SCNT and PA embryos that PZM-3 improved embryonic development to the blastocyst stage compared to NCSU-23 (Im *et al.,* 2004). In these previous studies (Yoshioka *et al.,* 2002; Im *et al.,* 2004), NCSU-23 did not contain essential and non-essential amino acids that were present in the PZM-3. It was not clear the low development of embryos that were cultured in NCSU-23 might be attributed to the absence of amino acids in NCSU-23. To clarify this difference in amino acids composition between PZM-3 and NCSU-23, we added the same concentrations of amino acids that were present in the PZM-3 to NCSU-23 and compared two media on embryonic development. Even though amino acids were added, NCSU-23aa could not increase embryonic development to the comparable level by mPZM-3, which suggested that components other than amino acids in mPZM-3, probably different composition of potassium and energy substrate, played an important role in improving developmental potential of pig embryos.

It has been reported that aged oocytes are more easily activated by parthenogenetic stimuli than younger oocytes (Ikeda and Takahashi, 2001). In addition, immature pig oocytes have different developmental potential depending on the originated follicles on the ovary. It has been demonstrated that oocytes from smaller follicles less than 3 mm in diameter show lower developmental capacity after PA, IVF, and somatic cell nuclear transfer than those from larger follicles (Yoon *et al.,* 2000; Wu *et al.,* 2006; Kim *et al.,* 2009). Thus, it was examined in this study whether the stimulating effect of mPZM-3 on embryonic development would be consistently shown even in PA oocytes that were activated at different time of IVM and that were derived from small and medium size follicles with different developmental competence. Irrespective of the time of activation after IVM and follicle size where oocytes were derived from, development to the blastocyst stage of PA pig oocytes were significantly improved by culturing in mPZM-3. Moreover, modification of NCSU-23aa by supplementing with myo-inositol, trisodium citrate, and β-ME increased blastocyst formation to some extent but the effect was not comparable to the same modification of PZM-3.

Our results obtained from a series of experiments showed consistently that mPZM-3 was more beneficial for embryonic development of pig PA oocytes than NCSU-23aa and mNCSU-23 and that the stimulating effect of mPZM-3 was consistently shown irrespective of the different methods for PA embryo production such as age of oocytes at the time of activation and source of oocytes. In summary, our results demonstrate that culture media have significant effect on preimplantation development PA embryos and that mPZM-3 is superior to mNCSU-23 in supporting embryonic development to the blastocyst stage in pigs. Further study is needed to clarify whether the beneficial effect of mPZM-3 would be shown consistently in the development of pig embryos that are derived from IVF and SCNT.

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