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Development of a Chemically Defined *In Vitro* Maturation System for Porcine Oocytes: Application for Somatic Cell Nuclear Transfer

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In the present study, performances of several *in vitro* maturation (IVM) systems for porcine follicular oocytes were evaluated, and an efficient chemically defined IVM system for porcine oocytes was proposed. The proposed one-step culture system supplemented with polyvinyl-alcohol (PVA) gave competitive efficiencies in terms of oocyte maturation and blastocyst development after parthenogenetic activation and *in vitro* culture, compared with the conventional two-step culture system by a supplementation of porcine follicular fluid (pFF). Additionally, it is identified that the proposed chemically defined one-step culture system yielded the comparable level of blastocyst production to the conventional maturation system in porcine somatic cell nuclear transfer (SCNT). Therefore, one can eliminate un-expected effects accompanied by supplementation of pFF. No medium replacement during whole maturation period is an additional benefit by applying this new system. Thus, these data support that the developed PVA supplemented chemically defined one-step IVM system for porcine follicular o ocyte might be used in porcine SCNT program.

Keywords: *In vitro* maturation, porcine, parthenogenesis, nuclear transfer

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Introduction

Incomplete cytoplasmic maturation as well as nuclear maturation leads to retardation of embryonic development. Therefore, considerable research efforts have been made to improve porcine *in vitro* maturation (IVM) system, and various IVM systems are developed. Among them, one of the most successful is the IVM system supplemented with porcine follicular fluid (pFF). The system is most widely utilized for porcine oocyte because of its superior maturation performance (Funahashi *et al.*, 1992). It is well-known that growth factors, hormones, and other factors, which are not defined yet, are abundant in serum or follicular fluid. Additionally, the components of follicular fluid are changed during follicular growth, and they affect metabolism of growing oocytes (Abeydeera *et al.*, 1998; Keskintepe *et al.*, 1995). Therefore, these undefined conditions make it difficult to control the quality and repeatability of IVM medium, as well as to examine the nutritional requirements of oocytes (Petters *et al.*, 1993).

All the above findings facilitate the endeavor to develop chemically defined IVM media. The first aim of these findings is eliminating and substituting of serum or follicular fluid. Considerable progress has been made in the past few years in this field. In the present study, polyvinylalcohol (PVA) supplemented chemically defined porcine IVM system (Table 1) was evaluated after parthenogenetic activation and *in vitro* culture: Development to the blasotcyst stage and the cell numbers in blastocysts were examined. Additionally, the optimal porcine IVM system chosen from the first series of experiment was applied to porcine somatic cell nuclear transfer (SCNT) program.

Materials and Methods

Chemicals

All inorganic and organic compounds were obtained from Sigma-Aldrich (Saint Louis, MO) unless otherwise stated.

In vitro maturation of porcine follicular oocytes

Slaughterhouse ovaries were collected from 5 to 6-month-old prepubertal gilts (110 ± 10 kg of body weight), placed in saline (30~35°C), and transported within 2 hours to the laboratory. After washing with saline three times, cumulus-oocyte complexes (COCs) were recovered by aspiration of 2 to 5-mm follicles using an 18-gauge hypodermic needle attached to a 5 ml disposable syringe. After washing with IVM medium three times, COCs enclosed by compact cumulus cells with more than three layers and an evenly granulated cytoplasm were selected for IVM. Selected COCs (50~100) were cultured in 4-well culture dishes (Nunc, Denmark) containing 500 μl of IVM medium under warmed and gas-equilibrated mineral oil for 22 hours at 38.5°C in 5% CO_2 in air. Subsequently, the oocytes were moved to fresh IVM medium without gonadotropins and incubated for an additional 22 hours. For one-step culture, some oocytes were remained in the first IVM medium, which included gonadotropins before parthenogenetic activation. The base medium for IVM was composed of tissue culture medium with Earl's salts and L-glutamine (Invitrogen, Carlsbad, CA) supplemented with 26.2 mM $NaHCO_3$, 3.05 mM glucose, 0.91 mM sodium pyruvate, 0.57 mM L-cysteine, 10% (v/v) pFF or 0.1% (w/v) PVA. The pFF was aspirated from superficial antral follicles of ovaries in prepubertal gilts, centrifuged at 3000×*g* for 10 min, filtered through a 1.2 μm syringe filter (Millipore, Bedford, MA), and stored in aliquots at -30°C until used.

Four IVM system grouped as follows: System 1) IVM for 44 hours in base medium supplemented with pFF and gonadotropins; System 2) IVM for 44 hours in base medium supplemented with PVA and gonadotropins; System 3) IVM for 22 hours in base medium supplemented with pFF and gonadotropins then for an additional 22 hours in gonadotropin-free medium; System 4) IVM for 22 hours in base medium supplemented with PVA and gonadotropins then for an additional 22 hours without gonadotropins (see Table 1 for details).

Parthenogenesis

Electrical activation was performed using an CF-150/B electro-cell fusion system (BLS, Hungary), at room temperature in a chamber with two stainless steel electrodes 1.0 mm apart, filled with activation buffer. Oocytes were activated with a pulse of 1.6 kV/cm DC pulse for 40 microsec. The activated oocytes were washed with culture medium three times and then treated with 5 μg/ml cytochalasin B containing North Carolina State University-23 medium (NCSU-23) for 5 hours.

Table 1. Summary of *in vitro* maturation systems for porcine follicular oocytes

Maturation conditions	[a]One-step culture		[b]Two-step culture	
	pFF	PVA	pFF	PVA
System 1	V			
System 2		V		
System 3			V	
System 4				V

[a]*In vitro* maturation for 44 hours in base medium with gonadotropins supplementation without medium replacement.
[b]*In vitro* maturation for 22 hours in base medium with gonadotropins then without gonadotropins for an additional 22 hours.
PVA: polyvinylalcohol; pFF: porcine follicular fluid.

Enucleation

Enucleation was performed with aspirating the oocytes using a beveled glass pipette with 30 μm outer diameter. With this treatment, first polar body (PB1) and the second metaphase plate in a small volume of surrounding cytoplasm were extruded from the cytoplast. Enucleated cytoplasts were then stained in Hepes-buffered NCSU-23 (HbN23) containing 5 μg/ml of Hoechst33342 for 5 min. Enucleation was confirmed by the absence of nucleus under ultraviolet light. After the enucleation, cytoplasts were washed extensively in HbN23.

Preparation of donor cells

The cells (porcine fibroblasts obtained from ear) starved by 0.5% fetal bovine serum (FBS) for 4 to 7 days were used for somatic cell nuclear transfer (SCNT). Immediately before injection, cell suspension of the donor cells was prepared by standard trypsinization. The cells were pelletted and resuspended in HbN23 and remained in this medium until the SCNT.

Nuclear transfer and activation

A beveled glass pipette with 15 μm outer diameter containing the donor cell was directly injected into the enucleated oocytes through cytoplast membrane. After the injection, the reconstructed embryos were remained in medium before activation. The activation of reconstructed embryos was same as the parthenogenetic one.

In vitro culture of parthenogenetic and SCNT embryos

All embryos were cultured in modified NCSU-23 (Roh *et al.*, 2002) for 7 days.

Statistic analysis

Average percentages were calculated from four independent experiments. Cleavage and further embryonic development among treatments were compared by chi-square test. Differences were considered statistically significant for P values <0.05.

Results

When cultured under chemically defined condition (System 2), medium replacement with hormone-free medium (System 4) beneficial for the nuclear maturation (71.4% *vs.* 56.1%; Table 2) and subsequent development to the blastocyst stage after parthenogenetic activation (15.8% *vs.* 5.9%; Table 3), whereas medium change showed better nuclear maturation (53.0% *vs.* 70.7%; Table 2) and blastocyst development (7.1% *vs.* 18.0%; Table 3) when matured in pFF supplemented medium (System 1 *vs.* System 3, respectively). There was no significant difference in cell numbers of blastocysts among all experimental groups (Table 3) after 7 days of *in vitro* culture.

Since blastocyst formation was significantly higher when System 2 and System 3 were used as IVM systems in porcine parthenogenesis program, both IVM systems were selected and applied to SCNT program. When the oocytes from these two IVM systems were used as recipient cytoplasts in SCNT, there was no significant difference in the rates of cleavage (43.8% *vs.* 48.5%) and blastocyst formation (8.5% *vs.* 10.3%) between two IVM systems (System 2 *vs.* System 3, respectively; Table 4).

Table 2. *In vitro* maturation of porcine follicular oocytes in a chemically defined culture system

Maturation conditions*	Number of oocytes	GV-GVBD	MI	MII (%)
System 1	164	24	53	87 (53.0)[a]
System 2	175	24	26	125 (71.4)[b]
System 3	157	16	30	111 (70.7)[b]
System 4	148	25	0	83 (56.1)[a]

[a,b]$p < 0.05$.
*See Table 1 for maturation conditions.
GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI/II: metaphase I/II.

Table 3. *In vitro* development of porcine parthenogenetic embryos matured in a chemically defined culture system

Maturation conditions*	Number of oocytes	2-cells (%)	Blastocysts (%)	Number of cells (±SE) in blastocysts
System 1	99	61 (61.6)	7 (7.1)[a]	33.8±2.1
System 2	101	64 (63.4)	16 (15.8)[b]	37.3±5.1
System 3	89	55 (61.8)	16 (18.0)[b]	40.5±7.5
System 4	101	56 (55.4)	6 (5.9)[a]	38.8±6.0

[a,b]$p < 0.05$.
*See Table 1 for maturation conditions.

Table 4. *In vitro* development of porcine nuclear transfer oocytes matured in a chemically defined culture system

Maturation conditions*	Number of oocytes	2-cells (%)	Blastocysts (%)
System 2	153	67 (43.8)	13 (8.5)
System 3	97	47 (48.5)	10 (10.3)

*See Table 1 for maturation conditions.

Discussion

In the present study, we found that viable SCNT embryos were produced in a chemically defined one-step culture system (System 2). Before confirming this IVM system, parthenogenetically activated porcine oocytes were cultured and 15% of blastocysts are obtained consistently. There is no significant difference in cell numbers of parthenogenetic blastocyst derived from four IVM systems. This result suggests that the development to the blastocyst stage is influenced by IVM system whereas the timing of blastocyst formation is not.

Krisher *et al.* (1999) reported that bovine blastocyst cultured in chemically defined conditions closely resembles in embryos from serum containing culture conditions when viewed by light microscopy. However, Keskintepe *et al.* (1995) showed that bovine embryos cultured in serum containing media appear dark and granular and blastocyst stage embryos cultured in chemically defined conditions resemble *in vivo* embryos. Similarly, porcine oocytes cultured in pFF containing media had dark and granular appearance.

Complex media with pFF as serum component contain many biological and chemical substances that are utilizable or inhibitory by oocytes and embryos (Bavister, 1995). Different batches of pFF, FBS, or bovine serum albumin (BSA) have variable effects and make media with pFF undefined occasionally. Effects caused by serum or BSA are a source of protein, chelation of heavy metals, introducing toxic agents or pathogens such as viruses, and binding other components of the medium. To avoid these unexpected effects of complex media, PVA has been used to replace serum or BSA (Bavister, 1981; Roh *et al.*, 2002). The substitution of PVA represents a pureness and refinement that eliminates viral or other infectious agents.

A chemically defined culture system is usually employed to find the specific components of media that affect the developmental competence (Liu and Foote, 1997) and to avoid various unknown interactions among medium supplements (Lee *et al.*, 2004; Hong *et al.*, 2004). Additionally, a chemically defined culture system will advance understanding of the basic molecular mechanisms responsible for stimulating or inhibiting nuclear and/or cytoplasmic maturation of pig oocytes (Abeydeera *et al.*, 1998). Since recipient cytoplasm plays a major role in the development of SCNT embryos (Roh, 2004), understanding of IVM mechanism is important for efficient production of transferable porcine SCNT embryos.

Our results may contribute to develop a chemically defined medium for the culture of porcine oocytes and provide a convenient one-step culture system without medium replacement. One-step culture lessens the chance for contamination occurred in media replacement occasionally and the change of culture conditions. The system may overcome many of the limitations of the conventional

IVM system containing pFF in the porcine. These results indicate that porcine follicular oocytes can be successfully matured under the chemically defined one-step culture system with subsequent development to the blastocyst stage after SCNT program.

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