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Effects of Oviductal Fluid, Culture Media and Zona Pellucida Removal on the Development of Porcine Embryos by Nuclear Transfer

Y. H. Zhang[1], E. S. Song[1], E. S. Kim[1], P. Q. Cong[1], S. H. Lee[1,2], J. W. Lee[3], Y. J. Yi[1] and C. S. Park[1,*]

[1] Research Center for Transgenic Cloned Pigs, Chungnam National University, Daejeon 305-764, Korea

ABSTRACT : The aim of this study was to compare the effects of oviductal fluid, porcine zygote medium (PZM)-3, PZM-4 and PZM-5, and modified PZM-5 culture media, and determine the effects of zona pellucida (ZP) removal on the development of nuclear transfer (NT) embryos. There were no significant differences in the rates of fusion and cleavage among the five different oviductal fluid concentrations. However, the rates of blastocyst formation and the cell numbers per blastocyst were high in the embryos at the 14 and 28 μg/ml concentrations of oviductal fluid compared to the 0, 56 and 100 μg/ml concentrations. The rates of cleavage and blastocyst formation, and the cell numbers per blastocyst were higher in the PZM-3, PZM-5 and modified PZM-5 media than in the PZM-4 medium. However, there were no significant differences in the fusion rates of oocytes among the four culture media. The cell numbers per blastocyst in the embryos without ZP were significantly greater than those with ZP. However, there were no significant differences in the rates of fusion, cleavage and blastocyst formation between the embryos with and without ZP. In conclusion, we improved blastocyst development and the quality of NT embryos by replacing PVA with 3 mg/ml of BSA in PZM-5 medium and supplementing the PZM-5 medium with 14 μg/ml oviductal fluid. The NT embryos produced by the zona-free NT method had a high rate of blastocyst formation in the modified PZM-5 medium. (**Key Words :** Oviductal Fluid, Nuclear Transfer, Culture Media, Zona Pellucida, Porcine)

INTRODUCTION

Important functions of the oviduct during reproduction include the provision of an optimal environment for gametes and zygotes and nutrition of the early embryos. These functions are ensured by the secretion of an oviductal fluid which is known to contain organ-specific glycoproteins. Several authors have identified oviduct-specific glycoproteins in the oviductal fluid of different species (Roberts et al., 1975; Boice et al., 1990; Gandolfi et al., 1991; Abe et al., 1993; Sendai et al., 1994) including the pig (Buhi et al., 1989). Oviductal fluid, secreted by the oviduct epithelium, provides a perfect environment for reproductive processes (such as oocyte maturation, capacitation of spermatozoa, and fertilization) and is essential for the nutrition of the early embryo (Leese, 1988). Some of these secretory components (glycoconjugates) were also demonstrated to associate with ovulated eggs or embryos in different species including golden hamsters (Abe and Oikawa, 1990), cows (Wegner and Killian, 1991) and pigs (Brown and Cheng, 1986; Hedrick et al., 1987).

NCSU-23 was the first medium to be used for efficient *in vitro* development of *in vivo* produced pig zygotes (Petters and Reed, 1991; Petters and Wells, 1993) and consequently it was widely used for culture of *in vitro* fertilization (IVF) and nuclear transfer (NT) embryos. However, full-term development of embryos cultured in NCSU was initially very low (Kikuchi et al., 1999). More recently, Kikuchi et al. (2002) modified its formulation with the addition of pyruvate and lactate and conditioning with oviduct cells and obtained the birth of IVF piglets after embryo transfer at the blastocyst stage. Yoshioka et al. (2002) evaluated the *in vitro* development of porcine zygotes that were cultured in a novel culture medium, porcine zygote medium (PZM), under different conditions and compared to *in vivo* development. A similar medium, called PZM-3 (Zhang et al., 2009), was used for embryo culture of both IVF and NT embryos and in the latter case it was found to be more efficient than NCSU-23 (Im et al., 2004) although the blastocysts obtained were not transferred into recipients.

* Corresponding Author: Chang-Sik Park. Tel: +82-42-821-5873, Fax: +82-42-822-6712, E-mail: parkcs@cnu.ac.kr

[2] College of Visual Image & Health, Kongju National University, Kongju, 340-702, Korea.

[3] Department of Animal Resource Science, College of Industrial Science, Kongju National University, Kongju, 314-701, Korea.

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Vajta et al. (2007) reported that the application of PZM-3 has contributed considerably to the improvement in the overall efficiency of the somatic cell nuclear transfer (SCNT) system. Yoshioka et al. (2002) demonstrated that the inner cell mass and total cell numbers in Day 6 embryos cultured in PZM-3, or in PZM-3 in which BSA was replaced with 3 mg/ml of polyvinyl alcohol (PZM-4), were also greater than those of NCSU-23 but less than those developed *in vivo*. Suzuki et al. (2002, 2004) developed PZM-5, in which the glutamine concentration was modified from 1 to 2 mM in PZM-4. The concentrations of free amino acids and the osmolalities in porcine oviductal and uterine fluids on day 3 and 5 after estrus were measured by Li et al. (2003). Based on these measurements they designed new media based on PZM-3 by modifying the amino acid composition and osmolality.

The major obstacle during the enucleation step is the zona pellucida (ZP). For this reason, different protocols have been devised for removal of the ZP from the oocytes prior to enucleation both for embryonic cloning (Peura et al., 1998) and SCNT (Booth et al., 2001ab; Vajta et al., 2001). However, this technique requires an efficient *in vitro* culture system to allow embryo development up to compacted morula or blastocyst, because only at these advanced stages can the zona-free embryos be transferred into recipients. Another advance in pig assisted reproduction has been the application of the zona-free method whereby zona-free oocytes have been fertilized and developed *in vitro* up to viable blastocysts capable of full- term development following transfer into recipient females (Wu et al., 2004). The technique of zona-free micromanipulation was applied successfully for the production of NT embryos and offspring in cattle and horses (Galli et al., 2003; Oback et al., 2003; Vajta et al., 2004; Lagutina et al., 2005). In the pig, this technique was developed for NT experiments (Booth et al., 2001b). Recently, Lagutina et al. (2006) reported the production of cloned pigs from zona-free NT blastocysts developed *in vitro* before transfer.

The aim of this study was to compare the effects of oviductal fluid, and PZM-3, PZM-4, PZM-5 and modified PZM-5 culture media, and determine the effects of ZP removal on the development of NT embryos.

MATERIALS AND METHODS

Collection and *in vitro* maturation (IVM) of porcine oocytes

Ovaries were collected from prepubertal gilts at a local slaughter house and transported to the laboratory in saline (0.9% (w/v) NaCl) at 30-35°C. Follicular fluid and cumulus-oocyte complexes (COCs) were aspirated from follicles of 2-6 mm in diameter using an 18-gauge needle fixed to a 10 ml disposable syringe. The follicular contents were pooled into 50 ml tubes and allowed to sediment. The sediment was placed into HEPES buffered Tyrode lactate medium (TL-HEPES-PVA; 114 mM NaCl, 3.2 mM KCl, 2 mM $NaHCO_3$, 0.34 mM NaH_2PO_4, 10 mM sodium lactate, 12 mM sorbitol, 2 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 10 mM HEPES, 0.2 mM sodium pyruvate, 65 μg/ml penicillin G and 25 μg/ml gentamycin sulfate) containing 0.1% (w/v) polyvinyl alcohol (PVA). Oocytes with uniform ooplasm surrounded by a compact cumulus cell mass were selected and washed with TL-HEPES-PVA and then washed twice with the maturation medium. The basic media used for *in vitro* maturation was modified tissue culture medium (TCM) 199 supplemented with 26.19 mM sodium bicarbonate, 3.05 mM glucose, 0.91 mM sodium pyruvate, 75 μg/ml sodium penicillin G, 50 μg/ml streptomycin sulfate and 0.1% (w/v) PVA. COCs were cultured in maturation medium containing 0.5 μg/ml luteinizing hormone (LH), 0.5 μg/ml follicle stimulating hormone (FSH), 10 ng/ml epidermal growth factor (EGF), 10% (v/v) porcine follicular fluids (pFF) and 0.57 mM cysteine. After 22 h of culture, oocytes were cultured without hormones for 22 h at 38.5°C, 5% CO_2 in air.

Preparation of porcine fetal fibroblast as donor cell

A porcine fetus on day 35 of gestation was obtained from a pregnant gilt. After the brain, intestines, and four limbs were removed, tissue was cut into small pieces with fine scissors. Cells were incubated for 30 min at 38.5°C in PBS containing 0.05% trypsin and 0.5 mM EDTA, and the suspension was centrifuged at 500×*g* for 20 min. The cell pellet was re-suspended and cultured in Dulbecco's Modified Eagle medium (DMEM, Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), 75 μg/ml penicillin G and 50 μg/ml streptomycin. The cells were frozen using DMEM supplemented with 10% (v/v) dimethyl sulfoxide (DMSO). To be used as donor cells in NT, cells were thawed and cultured until they reached confluence. Before NT, cells were treated with 0.05% trypsin and 0.5 mM EDTA for single-cell isolation at 2-5 min in a 38.5°C incubator. After washing with PBS, cells were re-suspended in manipulation medium (TCM199 supplemented with 0.6 mM $NaHCO_3$, 3.15 mM HEPES, 30 mM NaCl, 60 μg/ml penicillin, 50 μg/ml streptomycin and 0.3% bovine serum albumin (BSA)).

Nuclear transfer (NT) and culture of oocytes

After IVM for 44 h, the cumulus cells were removed from the oocytes by addition of manipulation medium supplemented with 0.1% (w/v) hyaluronidase. For micromanipulation, oocytes and donor cells were placed in a 50 μl drop under oil of manipulation medium

supplemented with 7.5 μg/ml cytochalasin B. Oocytes were enucleated by removing the first polar body along with adjacent cytoplasm containing the metaphase plate using a glass micropipette with an inner diameter of 20 μm. Through the same hole created in the ZP during enucleation, a cell was then placed in contact with the cytoplasm of each oocyte to form a couplet. After manipulation, couplets were washed once and equilibrated in TCM199 for 2 h at 38.5°C, 5% CO_2 in air before fusion and activation. Fusion/activation was accomplished with 1 DC pulse of 1.2 kV/cm for 30 μsec provided by a BTX Electro-cell Manipulator 200 (BTX, San Diego, CA, USA). Fusion medium was 0.3 M mannitol supplemented with 1.0 mM $CaCl_2 \cdot 2H_2O$, 0.1 mM $MgCl_2 \cdot 6H_2O$ and 0.5 mM HEPES. After fusion and activation, the reconstructed embryos were immediately cultured in PZM-3, PZM-4, PZM-5 and modified PZM-5 media at 38.5°C in a humidified atmosphere of 5% CO_2 depending on experimental designs, respectively. The rates of embryos at the cleavage and blastocyst stages were evaluated at 48 h and 6-7 days after activation, respectively.

Evaluation of embryo and blastocyst produced *in vitro*

Embryos and blastocysts were fixed with 2% formaldehyde for 40 min at room temperature, washed with PBS three times, permeated with PBS containing 0.1% Triton-X for 40 min at room temperature, and stained with 2.5 μg/ml DAPI (Molecular Probes, Eugene, OR, USA) for 40 min. Cell number per blastocyst was counted under an epifluorescence microscope (Olympus, Korea).

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

Experimental design

Experiment 1 was designed to investigate the effect of oviductal fluid on development of embryos produced by NT of porcine fetal fibroblasts. Estrus of a gilt was detected every 12 h from 22 weeks of age. Occurrence of estrus was defined by the standing reflex in front of a boar and reddening and swelling of the vulva. At day 2 of estrus, the animals were slaughtered and oviducts were flushed with 10 ml PBS to obtain oviductal fluids. Oviductal fluids were centrifuged at 1,000×*g* for 20 min, the supernatant filtered by 0.22 μm pore syringe filter (Adventec, Japan), its concentration measured by UV/Visible spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences, USA), and stored at -20°C. Various concentrations of oviduct fluids (0, 14, 28, 56 and 100 μg/ml) were added in PZM-5 culture medium.

Experiment 2 was carried out to investigate the effect of culture media on development of embryos produced by NT of porcine fetal fibroblasts. Porcine oocytes derived from NT were cultured in PZM-3, PZM-4, PZM-5 and modified PZM-5 media.

Experiment 3 was performed to examine the effect of ZP removal after fusion/activation on development of embryos produced by NT of porcine fetal fibroblasts. ZP was removed with 0.5% protease in TL-HEPES-PVA medium after fusion/activation. Oocytes without ZP were cultured in modified PZM-5 culture medium for further culture.

Statistical analysis

Analyses of variance (ANOVA) were carried out using the SAS package in a completely randomized design. Duncan's multiple range test and student's t-test were used to compare values of individual treatments when the F-value was significant ($p<0.05$).

RESULTS

Effects of oviductal fluid on the development of NT embryos

Five different concentrations of oviductal fluids were added into PZM-5 culture medium to investigate the development of embryos produced by NT of porcine fetal fibroblasts (Table 1). There were no significant differences in the rates of fusion and cleavage among five different oviductal fluid concentrations. However, the rates of blastocyst formation and the cell numbers per blastocyst were higher in the embryos at the 14 and 28 μg/ml concentrations of oviductal fluid compared to the 0, 56 and

Table 1. Effect of oviductal fluid concentration on development of embryos produced by NT of porcine fetal fibroblast[1]

Concentrations of oviductal fluid (μg/ml)[2]	No. of oocytes	% of oocytes fused[3]	% of oocytes cleaved[3]	% of blastocysts[3]	Cell no. per blastocyst[3]
0	145	85.0±3.8	73.5±3.7	14.1±1.8^{c}	31.9±1.9^{b}
14	147	92.1±2.6	84.7±0.5	27.6±1.9^{a}	41.1±2.4^{a}
28	140	90.1±1.9	85.5±3.7	26.2±2.4^{ab}	34.7±1.8^{ab}
56	148	90.6±3.1	82.6±3.5	18.1±3.4^{bc}	30.5±3.0^{b}
100	141	88.9±4.4	78.8±5.6	19.2±4.4^{bc}	30.9±3.4^{b}

[1] Donor cells were used from 3 to 6 passages. [2] Oviductal fluids were added into PZM-5 culture medium.

[3] Mean±SE. Experiments were repeated 6 times.

[a, b, c] Values in the same column with different superscripts differ significantly ($p<0.05$).

Table 2. Effect of culture medium on development of embryos produced by NT of porcine fetal fibroblast[1]

Medium[2]	No. of oocytes	% of oocytes fused[3]	% of oocytes cleaved[3]	% of blastocysts[3]	Cell no. per blastocyst[3]
PZM-3	143	92.8±1.6	73.7±2.1^{ab}	22.6±2.3^{ab}	34.2±1.3^{a}
PZM-4	144	92.0±1.9	69.4±1.2^{b}	11.8±1.9^{c}	25.7±4.1^{b}
PZM-5	146	93.6±1.4	75.1±2.5^{ab}	17.9±1.4^{b}	31.7±1.8^{ab}
Modified PZM-5	145	93.0±1.1	76.7±2.2^{a}	25.1±2.4^{a}	37.3±2.4^{a}

[1] Donor cells were used from 3 to 6 passages.
[2] Oocytes derived from NT were cultured in PZM-3, PZM-4, PZM-5 and modified PZM-5 media, respectively.
[3] Mean±SE. Experiments were repeated 6 times.
[a, b, c] Values in the same column with different superscripts differ significantly ($p<0.05$).

Table 3. Effect of ZP removal[1] after fusion/activation on development of embryos produced by NT of porcine fetal fibroblast[2]

Treatment	No. of oocytes	% of oocytes fused[3]	% of oocytes cleaved[3]	% of blastocysts[3]	Cell no. per blastocyst[3]
With ZP	156	93.1±1.7	76.6±3.8	23.9±2.7	30.4±3.0^{b}
Without ZP	156	91.5±2.2	74.4±3.1	23.5±2.6	41.5±3.7^{a}

[1] ZP was removed with 0.5% protease after fusion/activation of NT. [2] Donor cells were used from 3 to 6 passages.
[3] Mean±SE. Experiments were repeated 6 times.
[a, b] Values in the same column with different superscripts differ significantly ($p<0.05$).

100 μg/ml concentrations.

Effects of culture media on the development of NT embryos

A total of 578 oocytes derived from NT of porcine fetal fibroblasts were cultured in PZM-3, PZM-4, PZM-5 and modified PZM-5 media (Table 2). There were no significant differences in the fusion rates of oocytes among the four culture media. However, the rates of cleavage of oocytes and blastocyst formation, and the cell numbers per blastocyst were higher in the PZM-3, PZM-5 and modified PZM-5 media compared to the PZM-4 medium.

Effects of ZP removal on the development of NT embryos

A total of 312 oocytes with and without ZP were cultured in modified PZM-5 culture medium to examine the development of embryos produced by NT of porcine fetal fibroblasts (Table 3). There were no significant differences in the rates of fusion, cleavage and blastocyst formation between the embryos with ZP and without ZP. However, the cell numbers per blastocyst in the embryos without ZP were significantly greater than those with ZP. The blastocysts with enclosed ZP and removed ZP after fusion/activation by NT of porcine fetal fibroblasts are shown in Figure 1.

DISCUSSION

A component of oviductal fluids, probably a glycosaminoglycan, is reported to positively affect bovine sperm capacitation and the acrosome reaction (Parrish et al., 1989). Also, incubation of hamster (Minami et al., 1988) or pig (Krisher et al., 1989) embryos in an isolated mouse oviduct culture system improves embryonic development *in*

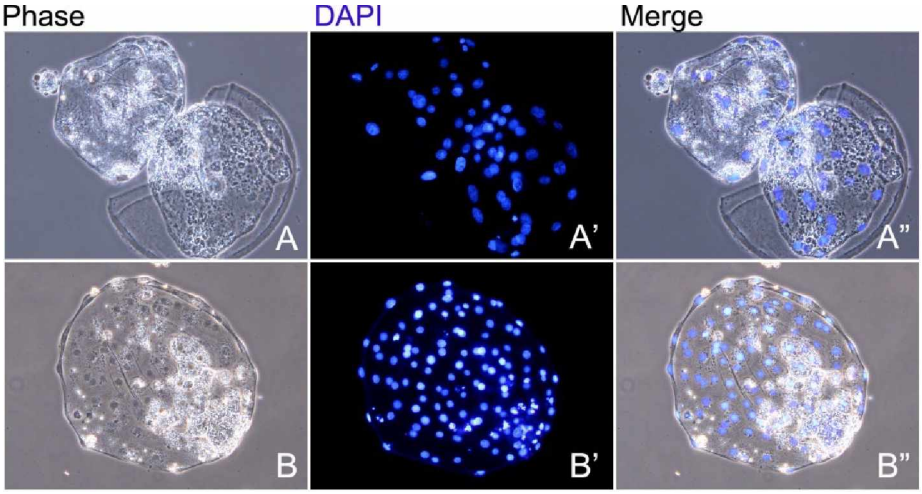

Figure 1. (A-A″) Blastocysts with enclosed ZP after fusion/activation by NT of porcine fetal fibroblast. (B-B″) Blastocysts with removed ZP after fusion/activation by NT of porcine fetal fibroblast.

vitro. Furthermore, it has been shown that an increased number of embryos will develop to blastocyst *in vitro* when incubated in culture medium previously conditioned by oviductal cells (Rexroad and Powell, 1988) or in medium supplemented with oviductal fluid (Archibong et al., 1989). Examination of the biochemical nature and biological function of the oviduct fluid would provide further study. In this study, the rates of blastocyst formation and the cell numbers per blastocyst were higher in the embryos at 14 and 28 μg/ml concentrations of oviduct fluid in PZM-5 culture medium.

We compared PZM-3 medium containing 3 mg/ml of BSA, PZM-4 medium replacing BSA with 3 mg/ml of PVA, PZM-5 medium altering the glutamine concentration from 1 mM to 2 mM in PZM-4 medium, and modified PZM-5 medium replacing PVA with 3 mg/ml of BSA in PZM-5 medium and supplemented with 14 μg/ml oviductal fluid. The results showed that the rates of oocyte cleavage and blastocyst formation, and the cell numbers per blastocyst were high in the PZM-3, PZM-5 and modified PZM-5 media compared to PZM-4 medium. Yoshioka et al. (2002) reported that replacement of fatty acid-free BSA with PVA in PZM had no detrimental effect on development of porcine zygotes up to day 6. Although the mechanism through which BSA affects embryo development is not yet fully understood, due in part to inclusion of numerous undefined impurities such as citrate and lipid transfer protein (Bavister, 1995), BSA may be beneficial for embryo culture because it can be taken up by embryos and broken down to provide amino acids for metabolic and anabolic processes as well as chelation of heavy metals or other toxic substances in the medium (Pemble and Kaye, 1986; Bavister, 1995). Suzuki et al. (2004) reported that PZM-5, in which the glutamine concentration was altered from 1 mM to 2 mM in PZM-4, improved blastocyst yield and the total cell number of blastocysts (Suzuki et al., 2002). The embryo can survive the high physiological osmolality *in vivo* because there are amino acids serving as "organic osmolytes", such as glycine (Dawson and Baltz, 1997), glutamine (Biggers et al., 1993), and β-alanine (Hammer and Baltz, 2003), in the female reproductive tract. The osmolytes glutamine and hypotaurine have been included in the PZM-3 formulation at 1 and 5 mM concentrations which have been shown to be effective in protecting against hyperosmolality (Dawson and Baltz, 1997). For *in vitro* culture of pre-implantation embryos, synthetic oviductal fluid (Tervit et al., 1972) and human tubal fluid (Quinn et al., 1985) media have been developed, which are based on the composition of sheep and human oviductal fluid, respectively. Yoshioka et al. (2002) developed a medium for *in vitro* culture of porcine zygotes based on the composition of pig oviductal fluid.

We investigated the development of the embryos with and without ZP. The results showed that the cell numbers per blastocyst in the embryos without ZP were significantly greater than those with ZP. The advantage of the zona-free method is in being more user-friendly than conventional micromanipulation but, on the other hand, this technique requires an efficient *in vitro* culture system to allow embryo development up to compacted morula or blastocyst, because only at these advanced stages can the zona-free embryos be transferred into recipients. Lagutina et al. (2006) reported for the first time that a cloned pig offspring was obtained following transfer of zona-free nuclear transfer blastocysts.

In conclusion, we improved blastocyst development and the quality of NT embryos by replacing PVA with 3 mg/ml of BSA in PZM-5 medium and supplementing the PZM-5 medium with 14 μg/ml oviductal fluid. The NT embryos produced by the zona-free NT method had a high rate of blastocyst formation in the modified PZM-5 medium.

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