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A comparison of co-culture with oviductal epithelial cells and growth on porcine zygote medium (PZM) on porcine embryos development

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Abstract- This study examined the effect of a chemically defined media system and co-culture system on the *in vitro* production of porcine embryos. Cumulus-oocyte complexes (COCs) from porcine ovaries were cultured for 22 h in TCM-199 with Earle's salts, L-glutamine, and $NaHCO_3$ supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml), 10 IU/ml of human chorionic gonadotropin, 25 µg/ml of follicle-stimulating hormone, 0.2 mM sodium pyruvate. But without the hormone for 22-44 h *in vitro* maturation (IVM) media. Matured oocytes were fertilized in basal IVF-media (modified Tris-buffered medium; mTBM). The porcine zygotes were randomly cultured in different culture conditions as follows: (1)a chemically defined media with porcine zygote medium (PZM), and (2) co-culture with the oviductal epithelial cells for embryo development. The results showed that the percentage of >4-cells, >16-cells developments and blastocysts in the PZM group (71.67%, 45.97%, and 24.33%, respectively) were higher than in the co-culture group (53.05%, 22.47%, and 8.85%, respectively) ($p<0.01$), but there was no significant difference between the co-culture group and PZM group for percentage of cleavage (70.00% and 71.43%, respectively) ($p>0.05$). These results indicated that *in vitro* production of porcine embryos using a chemically defined medium significantly increased the percentage of >4-cells, >16-cells development, and the blastocyst stage.

Keywords: Porcine embryo, *In vitro* production, a chemically defined medium system, co-culture system

1. Introduction

Porcine models have been used in medical research due to the genetic, anatomical, and physiological similarities to humans. Moreover, Pig models are used for the genetic improvement of herds and animal production. Numerous techniques, such as the establishment of an *in vitro* embryo production (IVEP) system, would be crucial for agricultural and biomedical purposes. A standard IVEP system is achieved through three technological steps: *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC). However, the efficiency of pig embryo IVP is still very low compared with *in vivo*-derived embryos (Romero-Aguirregomezcort *et al.*, 2021). There are many factors influencing the success of the procedure. One of the problems for embryonic development is suboptimal conditions for embryo culture (Poniedzialek-Kempny, 2020). The optimal medium conditions are an important factor influencing the quality and number of porcine embryos derived *in vitro*. In this work, the most commonly used embryo culture media were studied in two culture systems, somatic cell co-culture system and a chemically defined medium system, in an attempt to improve *in vitro* culture systems. Epithelial were used cells to support embryo development, which closely mimics the *in vivo* conditions. Porcine oviductal cells and their secretions, are involved in the provision of nutrients, detoxification, and production of antioxidant systems (Chen *et al.*, 2013; Heidari *et al.*, 2013). Recently, a chemically defined medium has been used for porcine embryo culture that supports the development of porcine zygote to blastocyst. For porcine embryos, culture media used are North Carolina State University medium (NCSU-23 or NCSU-37), Modified Calot, Ziomek, Bavister medium (CZB), Beltsville embryo culture medium (BECM-3), porcine zygote medium (PZM), and synthetic oviductal fluid medium (SOF) (Katska-Ksiazkiewicz, 2006; Yoshioka *et al.*, 2008; Spricigo *et al.*, 2019; Chen *et al.*, 2021). A chemically defined medium is the source of energy and amino acids. Moreover, a chemically defined medium system may decrease the risk of contamination by pathogens and viruses (Bruyere *et al.*, 2013; Mito & Hoshi, 2019). Therefore, it is essential to develop the medium for IVEP of porcine embryos, based on the improvement of medium components using for IVEP system. The objective of this study was compare co-culture with oviductal epithelial cells and porcine zygote medium (PZM) for the development of IVEP of a porcine embryo. In addition, we test the hypothesis that the establishment of a chemically defined medium system based on the composition of porcine oviductal fluid could support IVEP of porcine blastocysts.

2. Materials and methods

2.1 Oocyte retrieval and *In vitro* Maturation (IVM)

Ovaries were collected from the Kalasin Municipality Slaughter House and transported to the Department of Veterinary Technology Laboratory in 0.9% NaCl solution at 37 °C within 2-3 h of slaughter. Cumulus-oocytes complexes (COCs) were recovered by slicing ovaries in Dulbecco's Phosphate Buffered Saline (DPBS; Thermo Fisher Scientific Inc., Grand Island, USA) with 2% fetal bovine serum (FBS; Thermo Fisher Scientific Inc., Grand Island, USA), Pen/Strep solution (Thermo Fisher Scientific

Inc., Grand Island, USA), penicillin (100 U/ml), streptomycin (100 µg/ml). After the COCs were washed twice with DPBS medium, only a compact cumulus cell mass was selected and cultured in maturation medium. The medium for IVM was composed of TCM 199 medium with Earle's salts, L-glutamine, and $NaHCO_3$ (SIGMA-ALDRICH, Merck KGaA, Darmstadt, Germany) supplemented with 10% (v/v) FBS (Thermo Fisher Scientific Inc., Grand Island, USA), Pen/Strep solution (Thermo Fisher Scientific Inc., Grand Island, USA), penicillin (100 U/ml), streptomycin (100 µg/ml), 0.2 mM sodium pyruvate (SIGMA-ALDRICH, Merck KGaA, Darmstadt, Germany). COCs were cultured for 22 h in maturation medium supplement with 10 IU/ml of human chorionic gonadotropin (hCG; Chorulon, Intervet, Netherlands), 25 µg/ml of follicle-stimulating hormone (FSH; Folltropin® - V, Bioniche Animal Health (A/Asia) Pty. Ltd, Australia) and also without the hormone for 22-44 h in IVM medium at 38.5 °C in 5% CO_2 in humidified air.

2.2 *In Vitro* Fertilization (IVF)

After 44 h of culturing the oocytes in IVM media, 15-20 expanded COCs were washed three times and then transferred into a fertilization drop (500 ul) of basal IVF-media (modified Tris-buffered medium; mTBM) containing 113 mM NaCl (SIGMA-ALDRICH, Merck KGaA, Darmstadt, Germany), 3 mM KCl (SIGMA-ALDRICH, Merck KGaA, Darmstadt, Germany), 20 mM tris (SIGMA-ALDRICH, Merck KGaA, Darmstadt, Germany), 11 mM glucose (SIGMA-ALDRICH, Merck KGaA, Darmstadt, Germany), 5 mM sodium pyruvate (SIGMA-ALDRICH, Merck KGaA, Darmstadt, Germany), 7.50 mM $CaCl_2.2H_2O$ (SIGMA-ALDRICH, Merck KGaA, Darmstadt, Germany), 1 mM Caffeine (SIGMA-ALDRICH, Merck KGaA, Darmstadt, Germany), 1 mg/ml bovine serum albumin (BSA; SIGMA-ALDRICH, Merck KGaA, Darmstadt, Germany), Pen/Strep solution (Thermo Fisher Scientific Inc., Grand Island, USA), penicillin (100 U/ml), streptomycin (100 µg/ml). For the preparation of sperm for IVF, fresh boar semen stored at 18 °C was prepared for sperm capacitation. 1 ml of semen was added into 10 ml of mTBM medium containing 113 mM NaCl, 3 mM KCl, 20 mM triss, 11 mM, 5 mM sodium pyruvate, 11 mM glucose, 7.50 mM $CaCl_2.2H_2O$, 100 U/ml penicillin, 100 µg/ml streptomycin and then centrifuged for 5 min at 600 g (twice). The motile sperm were selected using the swim-up technique. 1 ml of mTBM was added into a 1.5 ml centrifuge tube and then the sperm pellet from the bottom of the was added and held for 1 h at 38.5 °C 5% CO_2. After 1 h of incubation, the top 0.8 ml from each tube was pooled in a 15 ml plastic centrifuge tube and centrifuged for 5 min at 600 g (twice). The final sperm pellet concentration in fertilization microdroplets was 2 X 10^6 sperm/ml. The oocytes and sperm were incubated for 12 h at 38.5 °C in 5% CO_2 (Brogni *et al.*, 2016).

2.3 *In Vitro* Culture (IVC)

After 12 h of incubation, fertilized oocytes were removed from cumulus cells by repeated pipetting of oocytes. Two IVC systems were used in this study; the oviductal epithelial cell co-culture system and PZM system. In oviductal epithelial cells co-culture system, fertilized oocytes were allocated to the co-culture. Porcine

oviductal epithelial cells were harvested from the lumen of the oviduct by flushing twice. The clumps of cells were collected into a sterile tube containing phosphate-buffered saline (PBS) and broken down with aspiration through an 18 gauge needle. Then the oviduct cells were centrifuged for 5 min at 600 g (twice). Finally, the pellet was seeded into a 35 mm Petri dish in TCM 199 supplemented with 10% (v/v) FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), 0.2 mM sodium pyruvate. The oviductal cells were cultured at 38.5 °C in 5% CO_2 in humidified air for 3-4 days, then 10 ul of the oviductal epithelial cells was transferred into 40 ul drop (5 embryos/drop) in a Petri dish and covered with mineral oil. In the PZM system, embryos were cultured in PZM containing 108 mM NaCl, 10 mM KCl, 0.35 mM KH_2PO_4, 0.40 mM $MgSO_4 \cdot 7H_2O$, 25 mM $NaHCO_3$, 0.20 mM sodium pyruvate, 2 mM Ca-$(Lactate)_2 \cdot 5H_2O$, 2 mM L-glutamine, 3.0 mg/ml BSA, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 ml/l MEM non-essential solution 100x (SIGMA-ALDRICH, Merck KGaA, Darmstadt, Germany), 20 ml/l BME essential solution 50x (SIGMA-ALDRICH, Merck KGaA, Darmstadt, Germany). Embryos were placed in a 50 ul drop (5 embryos/drop) in a Petri dish and covered with mineral oil and cultured in a humidified atmosphere of 5% CO_2 in an incubator at 38.5 °C. The embryos were cultured and the medium replaced every 48 h. Porcine embryos were cultured in the PZM system from day 0 to day 4, and then in IVC-Glucose (3 mg/ml; SIGMA-ALDRICH, Merck KGaA, Darmstadt, Germany) until day 8. All embryos were evaluated every 48 h to monitor development (Castillo-Martin *et al.*, 2012)

2.4 Experimental design

For evaluation of the effect of different culture systems on the production of porcine embryos *in vitro*, 420 COCs were cultured in IVM media. After 44 h of culturing in IVM media, expanded COCs were cultured with sperm in TBM. The presumptive zygotes were wash in culture medium in both the co-culture system and PZM. Then, zygotes were randomly allocated into different culture systems as follows. (1) co-culture with oviductal epithelial cells (n = 147), (2) PZM (n = 148). The embryonic development was evaluated every 48 h.

2.5 Statistical analysis

The comparison of different culture systems was analyzed with student's t-test, and using SAS software. A p-value of less than 0.01 was considered statistically significant.

3. Results

The study of different media showed that the percentage of >4-cells, >16-cells developments, and blastocysts in the PZM group (71.67%, 45.97%, and 24.33%, respectively) were higher than the co-culture group (53.05%, 22.47%, and 8.85%, respectively) ($p<0.01$), but there were no significant differences for the percentage of cleavage, between the co-culture group (70.00%) and PZM group (71.43%) ($p>0.05$) (Table 1). As shown in Figure 1, >4-cells, >16-cells, early blastocysts and blastocysts were observed after IVC.

Table 1. Effect of different culture systems on porcine embryo development in vitro

Types of culture system	No. of oocytes examined	No. of cleavage (% mean ± S.E)	No. of >4 cells (% mean ± S.E)	No. of >16 cells (% mean ± S.E)	No. of blastocyst (% mean ± S.E)
co-culture	210	147 (70.00±0.67)	78 (53.05±0.45)[a]	33 (22.47±1.07)[a]	13 (8.85±0.56)[a]
PZM	210	148 (71.43±0.78)	106 (71.67±1.36)[b]	68 (45.97±0.92)[b]	36 (24.33±0.96)[b]

[a, b] The values bearing different superscript within a column differed significantly $(p<0.01)$.

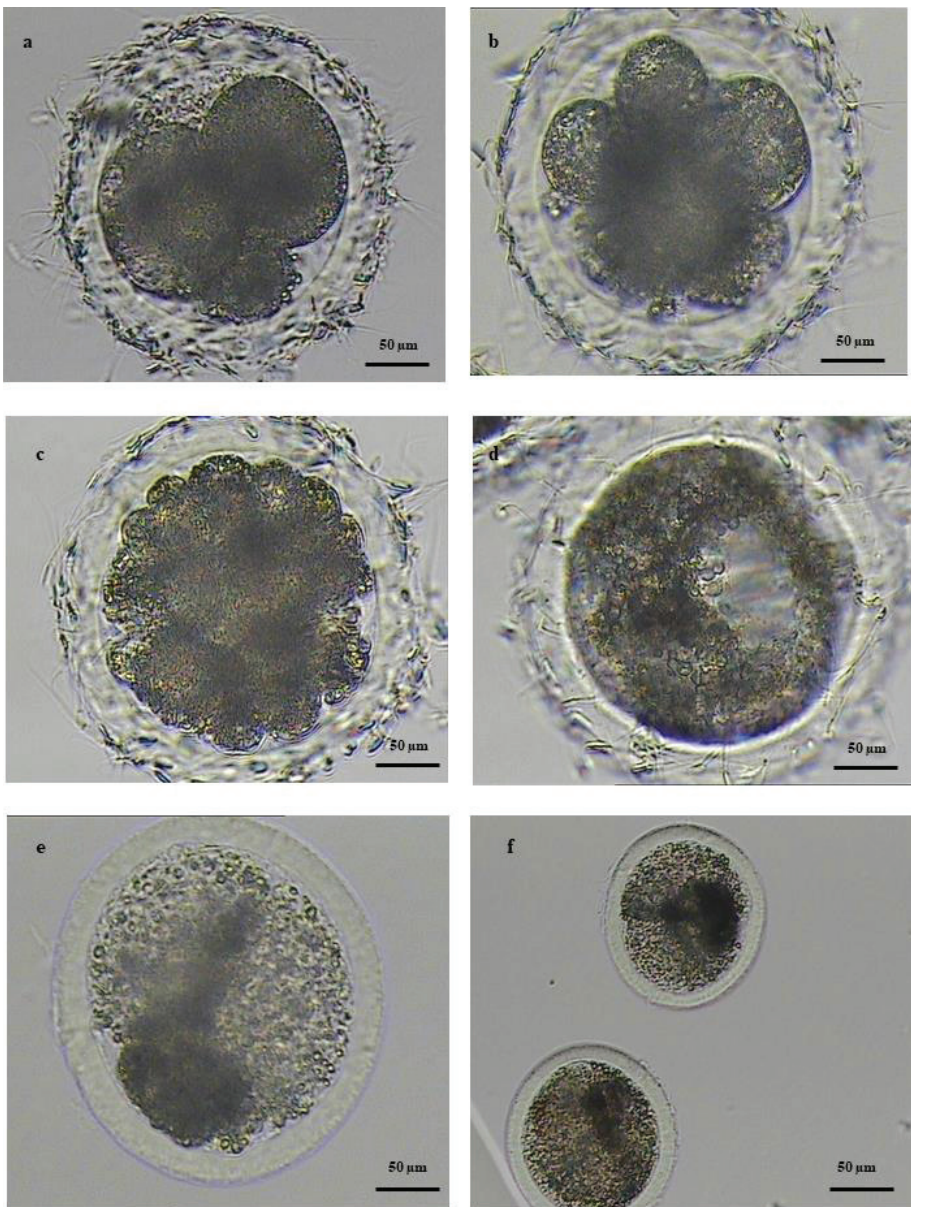

Figure 1. Representative image of porcine embryos produced in the PZM group. (a) 2-3 cells at 60 h after IVC; (b) >4-cells at 96 h after IVC; (c) >16-cells at 120 h after IVC; (d) early blastocyst at 156 h after IVC; (e-f) blastocyst at 168 h after IVC.

4. Discussion and conclusion

IVP of porcine embryos has become a technology that has been used as human bio models, for genomic selection, and for system biology (Motta *et al.*, 2018). The studies have shown that successful large-scale IVP of the porcine embryos can use to decrease cost and time, when compared to porcine embryos obtained *in vivo*. Oviduct epithelial cell co-culture system and a chemically defined medium system have been commonly used for *in vitro* culture (Carvalho *et al.*, 2017; Lee *et al.*, 2018). This study aimed to compare the co-culture system and a defined system for the development of IVP of a porcine embryo. The results showed the percentage of >4-cells stage and >16-cells stage in co-culture group was significantly lower than in the PZM group ($p<0.01$). Most of embryos were blocked at the 8-16 cells stage. The oviduct epithelial cells co-culture of the embryos did not support blastocyst formation. These results are similar to a previous study of the co-culture with oviduct epithelial cells (Smith *et al.*, 1992). Although it has been reported that oviduct epithelial cells have an effect in promoting blastocyst formation, our results from the co-culture system with porcine oviduct epithelial cells was limited for embryo development, similar to the previous report in co-culture with a various somatic cell such as mesenchymal stem cell (MSCs) and embryonic fibroblast cells (EFCs) was limited to the first 3 days of culture (Heidari *et al.*, 2013). Moreover, the higher percentage of the blastocysts at day 8 in the PZM group (24.33%) compare to the co-culture group (8.85%) in this study, indicated that the cell-free culture system was improved, in terms of embryos development. These results are similar to the present study that using a chemically defined medium could increase the production of good quality embryos and blastocyst development (Duarte *et al.*, 2020). The addition of a macromolecular component such as BSA or PVA to a chemically defined culture medium supports the development of embryos. Furthermore, the addition of amino acids to PZM plays an important role as heavy metals chelators and energy substrates. Moreover, amino acids may decrease the accumulation of H_2O_2 and protect the embryos from oxidative stress during IVC (Suzuki *et al.*, 2007). Glucose is also necessary even at an early stage, for the maintenance of viability of preimplantation embryos. In swine, a chemically defined medium was available for the successful culture of embryos to the blastocyst stage (Dang-nguyen *et al.*, 2020; Poniedzialek-Kempny, 2020), although the percentage of blastocyst in the co-culture system was lower than in a chemically defined medium *in vitro* embryo production. This may be caused by somatic cells which might secrete unknown cytokines and growth factors, resulting in inadequat support for embryo development (Yoshioka, 2011).

Our results showed that the percentage of >4 cells, >16 cells development, and blastocyst stage were better supported by the chemically defined medium system compared to the co-culture system. These results suggest that the chemically defined medium system can be used for the *in vitro* production of porcine embryos. However, future studies are required to improve both the chemically defined medium system and the co-culture system with various sources of somatic cells *in vitro* produced porcine embryos.

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Ethical statement: Experimental procedures were approved by the Committee on the Ethics of Animal Experiments of Kalasin University (Approval Number: KSU-AE-004/2022).

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