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Assisted Reproduction

Effects of type and state of co-culture cells on in-vitro development of porcine oocytes matured and fertilized in vitro

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***Purpose*:** The present study was to investigate the impact of type and state of co-culture cells on developmental competence of porcine oocytes matured and fertilized in vitro.
***Methods*:** Porcine zygotes were co-cultured with granulosa cells (GCs) (Group 1) or porcine oviductal epithelial cells (pOECs) at follicular stage (Group 2), ovulation stage (Group 3) or corpus luteum (CL) stage (Group 4) or cultured in a medium without co-culture cells (control group).
***Results*:** The proportion of oocytes developed to 2-cell stage embryos in Group 2 was similar to that in control group, but significantly ($p < 0.05$) lower than that in Groups 1, 3 and 4. The proportions of oocytes developed to ≥4-cell stage embryos in Groups 3 and 4 were significantly ($p < 0.05$) higher than that in Groups 1 and 2. At 144 h after insemination, 12.0, 14.8 and 20.0% of oocytes developed to blastocysts in Groups 1, 3 and 4, respectively. However, no embryos in control group developed beyond 4-cell stage and no embryos in Group 2 developed to blastocyst stage.
***Conclusion*:** As compared with GCs and pOECs at follicular stage, the pOECs at ovulation and CL stages had a better competence to support porcine embryo development under in vitro conditions.

KEY WORDS: Co-culture; granulosa cells (GCs); in-vitro development; porcine embryos; porcine oviductal epithelial cells (pOECs).

INTRODUCTION

The objective of in vitro culture of human and animal embryos is to provide high quality embryos capable of continuing development and implantation, and resulting in viable births (1). The development of specific culture regimes capable of supporting in vitro maturation (IVM), in vitro fertilization (IVF) of oocytes and subsequent development to the blastocyst stage has continued unabated in recent years (2). Although some authors have reported successful pregnancies and live births after transfer of porcine 2–4-cell stage embryos resulted from IVM–IVF, the developmental potential of in vitro-produced embryos is still lower than that of their in vivo counterparts (2–4). Low blastocyst development was also reported when in vivo matured oocytes were inseminated and culture in vitro (5). Furthermore, some embryos resulted from IVM–IVF were able to develop to blastocyst stage in vitro, but they could not establish pregnancy after transfer to recipients (6,7). The possible reasons for these are due to suboptimal embryo culture conditions, thus these embryos have low viability.

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Improvement on in vitro embryo production (IVP) system may rely on further understanding of embryonic metabolic requirements and better imitation of the developmental conditions in vivo (8). The mammalian oviduct provides the physiologic condition for early embryo development in vivo, and plays a critical early role in the establishment of a successful pregnancy (9). Recently, the culture system of porcine embryos has been greatly improved and several IVP systems including co-culture with porcine oviductal epithelial cells (pOECs) or granulosa cells (GCs) have been developed (10–12). It would appear that the co-culture system with pOECs or GCs could increase embryonic development. However, the embryos used in the previous studies were mainly derived from in vivo one- or two-cell embryos or produced by IVF of oocytes matured in vivo. However, embryos produced by IVM–IVF were not used in the co-culture system. Furthermore, the pOECs in different reproductive cycles may have different secretory activities, and the secretion from these cells may also have different components or different concentrations of each component (9,13).

The objectives of the present study were to investigate the effects of type and status of co-culture cells on the in-vitro development of porcine embryos resulted from IVM–IVF.

MATERIALS AND METHODS

Chemicals

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

Collection of Follicular Oocytes and IVM

Ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory within 2 h in saline containing 100 IU penicillin GK/mL and 100 IU streptomycin sulphate/mL at 37°C–38°C. Cumulus-oocyte complexes (COCs) were aspirated with a 10-mL syringe equipped with a 19-gauge needle. Only oocytes with compact cumulus cells were selected and washed three times in Dulbecco's-PBS (D-PBS, Cat. No. D6434) supplemented with 5% newborn calf serum (NCS, Gibco, Cat. No. 16010-159, pH: 7.48). COCs were then washed three times with IVM medium and transferred to a droplet of IVM medium (10–15 COCs/100 μL) under mineral oil (Cat. No. M5310) in a polystyrene dish (35 mm, Nunclon, Denmark, Cat. No. 153066). The basic medium used for IVM was Tissue Culture Medium 199 (TCM199; Gibco, Grand Island, NY; Cat. No.31100-035) supplemented with 10% fetal calf serum (FCS, Gibco, Cat. No. 16000-036), 10 IU/mL eCG (Ningbo, China, Cat. No.981018), 10 IU/mL hCG (Ningbo, China, Cat. No. 980630), 1 μg/mL estradiol-17β(Fluka, Switzerland, EEC No. 2000238), 100 mg/L sodium pyruvate, 900 mg/L calcium lactate, 550 mg/L D-glucose, 5958 mg/L Hepes, 100 IU/mL penicillin GK, and 100 IU/mL streptomycin sulphate (pH: 7.38). The COCs were cultured for 36 h at 39°C in a CO_2 (5% CO_2 in air) incubator (Asahi, Japan, Model 4020). After maturation, oocytes with expanded cumulus mass were selected for insemination.

Sperm Preparation and In Vitro Fertilization

Frozen-thawed epididymal spermatozoa were used in the present study. The methods for freezing the spermatozoa were previously described by Qian *et al.* (14). Briefly, boar epididymides (Landrace × Meishan × Yorkshire, 8 month-old) were removed at the slaughterhouse and returned to the laboratory in saline at 37°C within 15 min. The epididymal spermatozoa were extruded from the distal portion of the caudal by pressure using a 20 mL syringe, then mixed with BF-3 solution (1:3, v/v) (BF-3 solution: 4 g/100 mL lactose, 2 g/100 mL casein, 2 g/100 mL Tris, 1 g/100 mL citric acid monohydrate, 1 g/100 mL sucrose) and kept for 2 h at 4°C. Finally, BF-3 solution containing 4% glycerol was added to the sperm suspension at 1:1 (v/v) ratio and this sperm suspension was cooled for 2 h at 4°C until they were frozen in 0.1 mL pellets on dry ice and stored in liquid nitrogen.

Frozen spermatozoa (three pellets) were thawed in 10 mL D-PBS containing 5% NCS, 2 mg/mL BSA-V (Bochringer, German) (pH: 7.48) at 37°C, and centrifuged for 4 min at 200 g. The supernatant was discarded and spermatozoa were subsequently diluted to 2–4 × 10^8 cells/mL and preincubated in TCM199 supplemented with 12% FCS, 90 mg/100 mL calcium lactate, 10 mg/100 mL sodium pyruvate, 55 mg/100 mL D-glucose, 595.8 mg/100 mL HEPES, 100 IU/mL penicillin GK, and 100 IU/mL streptomycin sulfate (pH: 7.80) at 37°C in a 5% CO_2 incubator for 1 h. After preincubation, the proportion of spermatozoa with progressive forward motility was more than 60%.

For IVF of oocytes, matured oocytes with expanded cumulus mass were washed three times and transferred to fertilization medium. The fertilization medium was BO medium supplemented with 10 mg/mL BSA-V (Bochringer, German) and 2 mM caffeine (Cat. No. C6035) (pH: 7.48). A portion of the preincubated sperm suspension was introduced into the fertilization medium to give the final concentration of 2–4 × 10^7 cells/mL. The dishes were returned to a 5% CO_2 incubator at 39°C until examination of fertilization and embryo culture.

Examination of Fertilization and Embryo Culture

At 12–16 h after insemination, oocytes were removed from the surrounding cumulus mass and spermatozoa by repeated pipetting with a narrow-bore glass pipette. For examination of fertilization, some oocytes were fixed for 3–4 days with acetic alcohol (methanol and acetic acid, 3:1, v/v) at 4°C, stained with 1% aceto-orcein, and examined under a phase-contrast microscope (Olympus, Japan). Oocytes having enlarged sperm head and/or male pronucleus with a sperm tail were regarded as normal fertilization. For embryo culture, subgroup of presumptive zygotes were washed twice with an embryo culture medium: TCM199 supplemented with 10% FCS, 3.7 mL/L sodium lactate (60% syrup), 40 mg/L sodium pyruvate, 5958 mg/L Hepes, 100 IU/mL penicillin GK, and 100 IU/mL streptomycin sulfate (pH: 7.40). Each 10–15 embryos were transferred to a droplet of embryo culture medium (100 μL) and cultured in a 5% CO_2 incubator at 39°C with or without co-culture cells. Embryo development was examined at 24 h (2-cell stage), 48 h (2- and 3–4-cell stage), 72 h (>4-cell stage) and 144 h (blastocyst formation) after culture.

Preparation of Co-Culture System

Preparation of Granulose Cells. Granulosa cells (GCs) were collected from COCs. Briefly, the oocytes with expanded cumulus mass were selected and treated with 0.1% hyaluronidase (Cat. No. H4272). After treatment, the GCs were washed 2–3 times with embryo culture medium, and then added into the embryo culture medium at a concentration of 1×10^5 cells/mL and cultured for 24 h to produce monolayers before embryo culture.

Preparation of pOECs. The porcine oviducts were collected from sows at a local abattoir and transported to the laboratory together with ovaries in saline with 100 IU/mL penicillin GK and 100 IU/mL streptomycin sulfate. The oviducts were immediately freed from the ovaries after examination and record of the follicular stages in the ovary. After washed three times in D-PBS, the oviducts were flushed with 10 mL of D-PBS supplemented with 5% newborn calf serum three to four times. The collected solution was centrifuged at 250 g for 5 min. The supernatant was discarded and the cell pellets were washed in the embryo culture medium. The pooled pOECs with specific follicular stages were resuspended in the embryo culture medium to give a final concentration of 1–2 × 10^5 cells/mL and cultured for 48 h in a CO_2 incubator at 37°C to produce monolayers.

Experimental Design and Statistical Analysis

Five culture systems were used to examine developmental competence of embryo resulted from IVM–IVF in this study. Group 1: Co-culture with GCs; Group 2: Co-culture with pOECs at the follicular stage in which the surface of associated ovaries were enriched with developing follicles; Group 3: Co-culture with pOECs at ovulation stage in which the corpus haemorrhagicum existed on the surface of ovaries; Group 4: Co-culture with pOECs at corpus luteum (CL) stage in which the surface of ovaries were occupied with CL. Group 5: Control group. The embryos were cultured in the culture medium without co-culture cells.

Statistics Analysis

Data were analyzed with χ^2-test using SAS system for windows. Differences were considered to be significant at $p < 0.05$.

RESULTS

When oocytes were examined at 12–16 h after insemination, it was found that 75.6% (34/45) of the oocytes were penetrated by spermatozoa. As 23.5% (8/34) of the oocytes were polyspermic penetration, 61.8% (21/34) of oocytes were fertilized normally, showing both male and female pronuclei.

When presumptive zygotes were examined at 24 h and 48 h after culture, it was found that the proportions of 2-cell embryos in Groups 1 (39.1%), 3 (37.5%) and 4 (36.2%) were significantly ($p < 0.05$) higher than that in Group 2 (16.2%), but did not have

significant difference as compared with control group (Group 5; 26.5%). When these 2-cell embryos were continued in the culture and examined at 48 h, it was found that there was no significant difference on the proportions of 3–4-cell embryos between Groups 1, 2, 3, and 4, and they were all significantly ($p < 0.05$) higher than that in control group. When these embryos were examined at 72 h, the proportions of >4-cell embryos in Groups 3 and 4 were significantly ($p < 0.05$) higher than those in Groups 1 and 2. No embryos in control group developed beyond 4-cell stage. When these embryos were continued examined at 144 h, it was found that 12.0, 14.8, and 20.0% of the embryos developed to blastocyst in Groups 1, 3, and 4, respectively, but no embryos in Group 2 developed to blastocyst stage (Table I).

DISCUSSION

Under in vivo conditions, early embryos develop in the maternal reproductive tract. The key to successful in vitro embryo culture is to mimic these in vivo micro-environments (1). The mammalian oviduct plays a critical role in establishment of a successful pregnancy, even though the gametes and embryos stay within the oviduct briefly (9). In order to mimic in vivo conditions, some commercial IVP systems of mammalian embryos use co-culture systems. These systems include oviductal epithelial cells, cumulus cells, granulose cells, and established cell lines (1). Several reports have shown that the use of co-culture results in better embryonic development, higher cleavage rate and blastulation, and increased pregnancy rate in some animals and human (15).

In most of species, there is a specific embryo development stage that is very sensitive to in vitro conditions. This stage is called "in vitro developmental block." In pig, this in vitro developmental block is at 4-cell stage and is correlated with the onset of significant amounts on RNA synthesis (16) and the apparent transition time from maternal to embryonic genetic control (17). Many previous studies have demonstrated that co-culture systems can significantly enhance in vitro developmental competence of porcine embryo by overcoming the "4-cell block." The present study also indicated that if pig embryos were co-cultured with GCs or pOECs, 18.2–55.6% embryos can develop beyond 4-cell stage. On the contrary, no embryos develop beyond 4-cell stage without co-culture cells. Current hypotheses by which co-culture is beneficial for embryonic development cover three main areas: (1) Helper cells (i.e. co-culture cells) produce mitogenic substances for the embryos; (2) extracellular matrix products from the helper cells promote embryonic cell differentiation; and (3) the helper cells can metabolize or sequester embryotoxic substances from the culture medium (18). The results from the present study support the above hypotheses that the co-culture system could improve the development of the embryo in vitro. The detailed mechanism(s) by which the co-culture system deserves the beneficial effect to embryo development remains further studies.

pOECs were generally recovered at embryo collection from the oviduct flushes of day-1 and day-2 (day 0: ovulation) embryo donors by many previous studies (19,20). It is worth noting that the state of these pOECs before recovery has been synchronized with the embryo development. It has been shown that the oviduct undergoes changes in its secretory processes, fluid volume, and concentrations of ions and organic constituents concomitant with secretory patterns of ovarian steroids (9,13). So the state of pOECs may be affected by the status of ovary. In the present study, based on the follicular development stages in the ovaries, we used different pOECs in our

Table I. Effects of Type and Status of Co-Culture Cells on Development of Porcine Embryos Derived from In Vitro Matured and Fertilized Oocytes

Group	No. of eggs examined	No. (%) eggs cleaved to 2-cell	No. (%) of 2-cell embryos developed to		
			3–4-cell	>4-cell	Blastocyst
1	64	25/64 (39.1)a	12/25 (48.0)c	6/25 (24.0)e	3/25 (12.0)
2	68	11/68 (16.2)b	7/11 (63.6)c	2/11 (18.2)e	0
3	72	27/72 (37.5)a	15/27 (55.6)c	15/27 (55.6)f	4/27 (14.8)
4	69	25/69 (36.2)a	14/25 (56.0)c	11/25 (44.0)f	5/25 (20.0)
5	68	18/68 (26.5)ab	3/18 (16.5)d	0	0

Note. Values followed by different letters differ.
$p < 0.05$.

co-culture systems. We found that the pOECs at ovulation stage and CL stage had a better competence to support the embryonic development, as compared with follicular stage. These results indicate that the status of pOECs may affect their ability to support in vitro development of porcine embryos in the co-culture system, and the pOECs at ovulation and CL stages may be more homochronous with the developmental stage of porcine embryos.

Furthermore, our results have also indicated that porcine embryos developmental capacity display potential differences when they are co-cultured with different types of cells. The present co-culture system with pOECs at ovulation and CL stages represented the more efficient in vitro co-culture system for the development of porcine embryos derived from IVM–IVF. Although it is very general that co-culture would influence early embryo development irrespective of cell types (21), some types of cells must have more specific influences than others. This phenomenon is very obvious when cells from reproductive tracts are assessed as these cellular functions are affected by reproductive cycles. This may be the reason that pOECs are capable of exerting an additional beneficial influence on the development of porcine embryos over the GCs.

In conclusion, the present study indicates that: 1) The developmental competence of porcine IVM–IVF embryos was significantly improved when co-cultured with GCs or pOECs; 2) the status of pOECs may be affected by the status of ovary, thus the pOECs at ovulation and CL stages had a better competence to support the embryonic development, especially beyond 4-cell, as compared with follicular stage; 3) porcine embryos developmental capacity displayed potential differences when co-cultured with different types of cells, and their competence developed beyond 4-cell stages was significantly higher when co-cultured with pOECs at ovulation and CL stages than with GCs.

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