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Original article

A new adaptation for *in vitro* co-culture of single porcine parthenogenetic embryos using feeder cells

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Abstract

Feeder cells can promote cell proliferation and help overcome the developmental arrest of early embryos by producing growth factors. The objective of this study was to evaluate the effects of feeder cells on the development of all single porcine parthenogenetic embryos *in vitro*. Firstly, we showed that the cleavage and blastocyst formation rate of all single procine parthenogenetic embryos co-cultured with feeder cells increased in contrast to those cultured without feeder cells ($p<0.05$). However, no statistically significant differences were observed between the blastocyst formation rate in the embryos co-cultured with 3 different kinds feeder cells namely oviduct epithelial feeder cells, granulose feeder cells and porcine fetal fibroblast feeder cells ($p>0.05$). Secondly, highly significant differences were observed between the cleavage and blastocyst formation rate ($p<0.05$) when the embryos were co-cultured with oviduct epithelial feeder cells in different volume drops ranging from 3 to 20 µL and the cleavage rate were the highest when cultured in 5 µL drops. Thirdly, the tempospacial pattern of the development of single embryos co-cultured with oviduct epithelial feeder cells was consistent with that of traditional multi-embryo culture, indicating that the co-culturing does not affect the developmental competence of the porcine parthenogenetic embryos. Finally, highly significant differences were observed between the cleavage and blastocyst formation rate with and without zona pellucida *in vitro* ($p<0.05$). In this study, a new adaption of *in vitro* co-culture of single porcine parthenogenetic embryos using feeder cells has been successfully established and this will facilitate further investigations to discover the mechanistic mode of developmental arrest of porcine embryos.

Key words: feeder cells, porcine, embryos, in vitro

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Introduction

In recent years, embryos produced *in-vitro* have been widely used for the production of economically valuable animals (Yang et al. 2018) and disease models (Whitworth et al. 2014, Liu et al. 2018). At the same time, the application of *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and embryo transfer has greatly promoted the development of animal science. However, the low developmental ability of embryos *in-vitro* has greatly limited the development and application of these new technologies. Although many advances have been achieved to improve the developmental ability of embryos by adding or reducing factors to the culture medium of mammalian early embryos cultured *in vitro* (Wang et al. 2002, Wang et al. 2006, Thanh et al. 2011), most embryos *in vitro* arrested at early stages and the underlying molecular mechanism remains largely undefined (Teperek et al. 2013, Ogura et al. 2014, Liu et al. 2016). In this study, we have developed a new approach using feeder cells for porcine parthenogenetic embryo *in vitro* co-culture, which has allowed us to keep track of the developmental fate of a single embryo precisely and has provided the leads for futher studies on the developmental arrest of porcine embryos.

Materials and Methods

Chemicals

Unless otherwise indicated, chemicals were purchased from Sigma Chemical Company (Sigma-Aldrich, Wuhan, China).

Collection of cumulus-oocyte complexes (COCs) and in-vitro maturation

Collection and maturation of COCs were conducted according to a previously described method (Zhou et al. 2015). Porcine ovaries were obtained from an abattoir affiliated with COFCO (China Oil and Food Import and Export Corporation) in Wuhan and transported to the laboratory in 0.9% NaCl supplemented with 100 IU/mL penicillin G and 100 IU/mL streptomycin sulphate at 30 to 35°C. Collections of COCs were made immediately from follicles with a diameter of 3 to 6 mm using a 10-gauge needle attached to a disposable 10-mL syringe and COCs were stored in Dulbecco's Phosphate Buffered Saline (DPBS) supplemented with 5% fetal bovine serum (FBS) (Bi et al. 2016). Only COCs with a uniform cytoplasm and surrounded by several layers of cumulus-cells were used for *in vitro* maturation (IVM).

Selected COCs were washed three times in DPBS supplemented with 5% FBS and three times in maturation medium (Medium 199; Gibco) supplemented with 10% (v/v) pig follicular fluid (PFF), 0.1% (w/v) polyvinyl alcohol, 3.05 mM glucose, 0.91 mM sodium pyruvate, 0.57 mM L-cysteine, 100 IU/mL streptomycin sulphate (Gibco), 100 IU/mL penicillin G (Gibco), 10 IU/mL PMSG (Ningbo Second Hormone Factory), and 10 IU/mL hCG (Ningbo Second Hormone Factory) (Abeydeera et al. 2000). Incubation conditions for COCs were 42 to 44 h in a 5-well dish at 39°C in a Submarine Incubation System with 100% humidity and 5% CO_2 in air.

The maturation medium was incubated for at least 3 h at 39°C in the Submarine Incubation System with 100% humidity and 5% CO_2 in air.

Electrical activation of oocytes and in vitro culture

After 42 to 44 h *in vitro* culture, oocytes were denuded of cumulus-cells by gentle pipetting in tissue culture medium 199 (TCM-199) hepes-buffered medium supplemented with 1 mg/mL hyaluronidase for 4 min. Oocytes with polar body I (pb I) were selected, washed three times with activation fluid supplemented with 0.3 M mannitol, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, and 0.05 mg/mL bovine serum albumin (BSA), and then activated by applying a single DC pulse of 1.5 kV/cm for 30 μ sec using a BTX Electro-Cell Manipulator 2001 (BTX Inc, San Diego, CA, USA) (Hua et al. 2011).

For *in vitro* development after activation, the embryos were washed three times with North Carolina State University Medium-23 (NCSU-23) containing 4 mg/ml BSA (Petters et al. 1993) and then cultured in the medium as described above. The embryos were then incubated at 39°C in the Submarine Incubation System with 100% humidity and 5% CO_2 in air. Cleavage and blastocyst formation rate were determined 2 and 7 days after activation, respectively.

Preparation of feeder cells

Preparation of granulose cells

The porcine granulose cells (GC) were obtained from follicles with a diameter of 4-6 mm without atretic changes and containing oocytes with normal morphological appearance as previously described (Opiela et al. 2015). The follicles were dissected from the ovary and individual follicles were completely trimmed from the remaining connective tissues. Nonatretic follicles were selected based on morphological criteria including translucency, lack of free particles, and the presence of blood vessels. The selected follicles were transferred

Table 1. Effects of different feeder cells on the development of single porcine parthenogenetic embryos *in vitro*.

Treatment group	Oocyte number	Cleavage rate (%)	Blastocyst formation rate (%)
Control	88	39 (44.3) [a]	0 (0) [a]
Oviduct epithelial feeder cells	78	56 (71.8) [b]	4 (7.1) [b]
Granulose feeder cells	67	46 (68.7) [b]	3 (6.5) [b]
Porcine fetal fibroblast feeder cells	84	51 (60.7) [c]	3 (5.9) [b]

Different superscripts within the same column denote significant differences ($p<0.05$).

into holding medium and opened by puncture. The follicular fluid was released into the medium along with the oocyte and surrounding cumulus cells. If the morphology of the COCs met the criteria the follicle was transferred into the dish with IVM medium and the GCs attached to the follicle wall were scraped manually into the medium.The solution obtained was vigorously mixed to obtain homogenous mixture of GCs. A 1/10 dilution in GC/IVM mixture in DPBS of GCs in a 10 uL volume was used to count the GC concentration in a hemocytometer. The number of cells in 1 mL was calculated using an average of X counts per mixture and the concentration adjusted to 3-5 × 10^6 granulosa cells/mL.

Preparation of porcine fetal fibroblast cells

Preparation of porcine fetal fibroblast cells were carried out according to the method that described in Chinese patent, Hubei White Pig Fibroblast Line.

Porcine fetal fibroblast cells were collected following hysterectomy at days 35 of pregnancy (estrus 5 Day 0). Fetuses were washed 6-8 times in DPBS supplemented with 1% penicillin/streptomycin. Samples of back and hip muscles were excised with scissors and tweezers. Fetal fibroblast cells were collected with incubation of the samples in 0.25% trypsin for 10-15 min followed by gentle pipetting. After tissue disruption, cells were centrifuged for 5 min at 50 × gravity to remove tissue debris and clumps (Lee et al. 2000). Cells were collected and incubated in the Submarine Incubation System with 100% humidity and 5% CO_2 in air with DMEM adding 10% FBS.

Preparation of oviduct epithelial cells

Sow oviducts were obtained from an abattoir affiliated with COFCO in Wuhan and the transport process was the same as that of ovaries (refer to 2.2). They were washed 6-8 times in DPBS supplemented with 1% penicillin/streptomycin and cut into 2 cm small lengths. Tubal epithelial cells were isolated by incubation of the oviduct in 0.25% trypsin for 1-2 min followed by gentle pipetting. Cells were collected by centrifation for 5 min at 150 × g and the supernatant aspirated. Cells were collected and incubated in the Submarine Incubation System with 100% humidity and 5% CO_2 in air with TCM-199 adding 10% FBS.

Co-culture of embryos with feeder cells

One day before transferring the embryos into a drop of culture medium with feeder cells, cells were collected from the flask by trypsinization and washing, and were subsequently seeded at a concentration of 0.5 × 10^2 cells per mL and the drop volume was designed according to experiments, with one drop for co-culture per one embryo. Half of the medium was exchanged before incubation of the embryos (Opiela et al. 2018) and changed half the medium every 48 h hours after co-culture with embryo.

Statistical analysis

The data were collected from at least three replicates with each treatment represented in all replicates. They were analyzed with the χ^2 test. A probability of $p<0.05$ was considered significant.

Results

Effects of different feeder cells on the development of single porcine parthenogenetic embryos in vitro

To determine the effects of feeder cells on the development of single porcine parthenogenetic embryos *in vitro*, single procine parthenogenetic embryos were co-cultured in 10 uL drops with 3 different feeder cells namely oviduct epithelial feeder cells, granulose feeder cells and porcine fetal fibroblast feeder cells. The results showed that feeder cells could help to overcome the developmental arrest of single procine parthenogenetic embryos and increased significantly the cleavage and blastocyst formation rate in contrast to the embryos incubated without feeder cells ($p<0.05$) (Table 1). However, no statistically significant differences between the blastocyst formation rate in the embryos co-cultured with 3 different kinds of feeder cells were observed ($p>0.05$) (Table 1).

Table 2. Effects of different volume drops of oviduct epithelial feeder cells on the development of single porcine parthenogenetic embryos *in vitro*.

Treatment group (μL)	Oocyte number	Cleavage rate (%)	Blastocyst formation rate (%)
3	71	29 (40.8) [a]	0 (0) [a]
5	60	45 (75) [b]	3 (6.7) [b]
10	58	40 (69) [c]	2 (5) [b]
15	62	37 (59.7) [d]	1 (2.7) [c]
20	61	32 (52.5) [e]	0 (0) [a]

Different superscripts within the same column denote significant differences ($p<0.05$).

Table 3. Effects of different methods on the development of porcine parthenogenetic embryos *in vitro*.

Treatment group	Oocyte number	2-cell rate after activated 24h (%)	Cleavage rate after activated 36h (%)	4-cell rate after activated 48h (%)	Blastocyst formation rate of 7d (%)
Single embryo	60	30 (50) [a]	45 (75) [a]	31 (51.7) [a]	2 (6.7) [a]
Traditional cultivation in 5-well dish	396	237 (59.8) [b]	352 (88.9) [b]	277 (69.9) [b]	40 (16.9) [b]
Traditional cultivation in droplet	98	56 (57.1) [b]	83 (84.7) [b]	58 (59.2) [c]	7 (12.5) [b]

Different superscripts within the same column denote significant differences ($p<0.05$).

Table 4. Effects of oviduct epithelial feeder cells on the development of porcine parthenogenetic embryos without zona pellucida *in vitro*.

Treatment group	Oocytes number	Cleavage rate (%)	Blastocyst formation rate (%)
Control	82	59 (72) [a]	5 (8.5) [a]
Embryo without zona pellucida	77	30 (39) [b]	1 (3.3) [b]

Different superscripts within the same column denote significant differences ($p<0.05$).

Effects of different volume drops of oviduct epithelial feeder cells on the development of single porcine parthenogenetic embryos in vitro

Based on the results presented in Table 1, we used oviduct epithelial feeder cells as feeder cells due to it is easy to prepare to test the effects of co-culture medium volume on the development of single porcine parthenogenetic embryos *in vitro*. Highly statistically significant differences between the cleavage and blastocyst formation rate were observed when embryos were co-cultured with oviduct epithelial feeder cells in different volume drops ($p<0.05$) (Table 2) and the cleavage rate reached the highest when cultured in 5 uL drops (Table 2).

Effects of different methods on the development of porcine parthenogenetic embryos in vitro

Based on the results shown in Table 1 and Table 2, we also used oviduct epithelial feeder cells as feeder cells to compare the tempospacial pattern of a single procine parthenogenetic embryo co-cultured with oviduct epithelial feeder cells in 5 uL drops and traditional multi-embryo co-culture including traditional culture in 5-well dish and traditional culture in droplet. The results showed that the tempospacial pattern of the development of a single procine parthenogenetic embryo co-cultured with oviduct epithelial feeder cells was consistent with that cultured in traditional multi--embryo co-cultured (Table 3).

Effects of oviduct epithelial feeder cells on the development of porcine parthenogenetic embryos without zona pellucida in vitro

Furthermore, we attempted to understand the effects of feeder cell on the development of single procine parthenogenitic embryos without zona pellucida *in vitro*. Based on results shown in the Table 1 and Table 2, we also used the oviduct epithelial feeder cells as feeder cells co-culture a single procine parthenogenetic embryos with and without zona pellucida in this experiment. Highly significant differences were observed in the cleavage and blastocyst formation rates between the groups with and without zona pellucida *in-vitro* ($p<0.05$) (Table 4).

Discussion

To provide research models to hasten the development of animal science, animal embryos with better

quality need to be prepared for *in vitro* experimentation. Despite of newly improved culture conditions, co-culture procedures and enriched media (Perker et al. 2015), growth arrest occurs in most embryos *in vitro* at early stages and the underlying molecular mechanism remains largely undefined (Teperek et al. 2013, Ogura et al. 2014, Hu et al. 2015, Liu et al. 2016). The efficiency of preparing animal models such as those of pig and rabbit (Chen et al. 2013, Xu et al. 2013) using somatic cell nuclear transfer is poor (Niemann et al. 2008). Although much research has been focused on developing *in-vitro* porcine oocyte maturation and embryo production systems, there has been little progress in improving the survival and development of embryos produced *in vitro* (Biswas et al. 2018).

Based on previous research (Liu et al. 2016), we developed a new approach using feeder cells for single porcine parthenogenetic embryo *in vitro* co-culture that allows us to keep track of the developmental fate of a single embryo precisely in this study. Lee et al (2015) also investigated the effect of human adipose tissue-derived mesenchymal stem cells (hAT-MSC) and human embryonic stem cells (hESC) as a 10% supplement used instead of BSA and serum on the development of porcine embryos. Liu et al (2007) showed that SOF and granulose cells adding NCSU-23 supplemented with FCS and porcine oviduct epithelial cell monolayer can improve the development of porcine parthenogenesis embryos. It has also been observed that co-culturing IVF embryos with oviduct cells can ameliorate developmental blocks in porcine embryos produced through IVF (Swanson et al.1996). Liu et al. (2016) established an efficient SCNT embryo biopsy culture system to obtain two- and four-cell-stage single blastomeres from mice SCNT embryos with ascertainable developmental fates. Our results demonstrated that feeder cells can promote the development of a signal embryo and there was highly significant difference in the blastocyst development when co-cultured with feeder cells.

In conclusion, our study established a new adaption of *in vitro* co-culture of single embryo culture using feeder cells and this will facilitate further investigations to discover the mechanistic mode of developmental arrest of porcine embryos.

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