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Parthenogenetic Activation of Porcine Oocytes and Isolation of Embryonic Stem Cells-like Derived from Parthenogenetic Blastocysts

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ABSTRACT : These experiments were carried out to optimize the parameters of electrical activation, methods of parthenogenetic activation and embryo culture *in vitro* and meanwhile to isolate embryonic stem cells-like (ESCs) derived from porcine parthenogenetic blastocysts (pPBs). These results showed that, as the electric field strength increased from 1.0 to 2.7 kV/cm, the cleavage rate of parthenogenetic embryos increased gradually but the rate of oocyte lysis was significantly increased when using 2.7 kV/cm field strength. The rate of cleavage in 2.2 and 2.7 kV/cm groups was significantly increased in comparison with that of the 1.0 kV/cm group. A voltage field strength of 2.2 kV/cm DC was used to investigate blastocyst development following activation with a single pulse of 30 or 60-μsec pulse duration. The optimum pulse duration was 30-μsec, with a blastocyst rate of 20.7%. Multiple pulses were inferior to a single pulse for blastocyst yield (8.0% vs. 29.9) ($p<0.05$). For porcine oocyte parthenogenetic activation methods, the rates of cleavage (79.0% vs. 59.8%) and blastocysts (19.4% vs. 3.4%) were significantly increased in electrical activation in contrast to chemical activation with ionomycin/6-DMAP ($p<0.05$). Rates of cleavage and blastocyst formation in NCSU-23 and PZM-3 embryo media were higher than those of G1.3/G2.3 serial culture media, but there was no significant difference among the three groups. The total cell number of blastocysts in PZM-3 embryo culture media containing 5 μg/ml insulin was significantly higher than that of the control (no insulin) (44.3±9.1 vs. 33.9±11.7). For isolation of PESCs-like, the rates of porcine blastocysts attached to feeder layers and ICM colony formation in Method B (nude embryo culture) were better than those in Method A (intact embryo culture). (**Key Words :** Parthenogenetic Activation, Oocyte, Embryonic Stem Cells, Porcine)

INTRODUCTION

Parthenogenesis, the process by which a single oocyte can develop without the presence of the male counterpart, is a common form of reproduction in insects (Vrana et al., 2003). Artifical stimuli, such as exposure to ethanol, ionophore A23187, ionomycin, or direct electric pulses, can elevate the cytoplasmic Ca^{2+} levels and cause mammalian oocyte activation (Bing et al., 2003). These activation treatments were commonly combined with protein synthesis inhibitors, such as cycloheximide, and phosphorylation inhibitors, such as 6-dimethylaminopurine (Loi et al., 1998; Liu et al., 1999). Different protocols of porcine oocyte parthenogenetic activation have been described by many researches (Cha et al., 1997; Liu et al., 1997; Grupen et al., 1999; Grupen et al., 2002; Fan et al., 2003; Zhu et al., 2003; Lee et al., 2004; Yi et al., 2005; Somfai et al., 2006; Hossein et al., 2007). However, these activation treatments seem unable to provide an adequate or full-valued activation (Wang et al., 1998). In addition, the low cell numbers of cultured NT, IVF, and parthenogenetic blastocysts (PBs) as compared to those of *in vivo* blastocysts probably reflects the inadequacies of *in vitro* culture systems (Bettauser et al., 2000). Up to now, isolation and culture of ESCs derived from porcine PBs (pPBs) has not been reported. This may be indicative of the inadequacy of the activation treatments and *in vitro* culture systems. How to improve activation of porcine oocytes and *in vitro* culture systems have become necessary questions to be solved.

Mouse and monkey parthenogenetic embryonic stem cells (PESCs) had been established respectively (Allen et al., 1994; Cibelli et al., 2002; Vrana et al., 2003). For gene imprinting, theoretically, some imprinting genes abnormally

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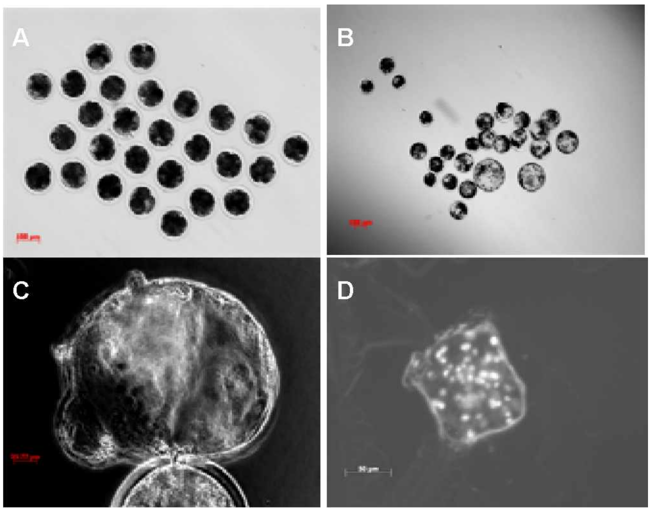

Figure 1. pPBs and blastocysts stained with Hoechst33342. A: 2-4 cells porcine parthenogenetic embryos ×100; B, C: pPBs, B ×50, C ×400; D: The total cell number of blastocysts were counted by Hoechst33342 staining ×400.

express in PESCs, which provides a good model for studying the role and mechanism of imprinting gene in ESCs proliferation and differentiation. Isolation and culture of porcine ESCs derived from fertilization *in vivo* or *in vitro* have been reported by some researchers using different culture conditions (Piedrahita et al., 1990; Strojek et al., 1990; Wheeler et al., 1994; Chen et al., 1999; Li et al., 2003) and some pluripotent characteristics of these porcine ESCs-like have been identified. But these researchers only employed short-time cell culture and stable ESCs lines similar to mouse and human ESCs lines have not been established. So far, to our knowledge, this is the first reported study to isolate and culture PESCs-like derived from pPBs.

In the present study, different electrical activation parameters, activation methods and different *in vitro* culture methods were compared to obtain a stable system of producing pPBs *in vitro*. Meanwhile, isolation and culture of PESCs-like from pPBs were initially investigated.

MATERIALS AND METHODS

Unless stated otherwise, all chemicals for this study were obtained from Sigma Chemical Company (St.Louis, MO).

Preparation of recipient oocytes derived from *in vitro* maturation culture

Porcine ovaries were obtained from a local slaughterhouse and transported to the laboratory in physiological saline at 30-37°C within 3 h after collection. Follicular fluid and cumulus-oocytes complexes (COCs) from 3-7 mm in diameter were aspirated using an 18-gauge needle attached to a 5-ml syringe. COCs with uniform cytoplasm and multi-layers of cumulus cells were selected and rinsed three times in PVA-TL-Hepes and in oocyte maturation medium, respectively. The oocyte maturation medium was modified TCM199 (adding 1 mg/ml PVA, 3.05 mM D-glucose, 0.91 mM Na-Pyruvate, 0.57 mM Cyseine) supplemented with 10 IU/ml PMSG, 10 IU/ml hCG, 2.5 IU/ml FSH, 10 ng/ml EGF, and 1% Insulin-Transferrin-sodium Selenite (ITS, Gibco). Approximately 50-70 COCs were transferred into each well of a four-well dish (Nunc, Roskilde, Denmark) containing 500 μl maturation medium. The oocytes were matured for 42-44 h at 38.5°C, in a humidified atmosphere of 5% CO_2. Following *in vitro* maturation, oocytes were denuded of cumulus cells by repeated pipetting in 0.3% hyaluronidase. Oocytes with the first polar body, an intact plasma membrane, round shape and visible perivitelline space were selected and used for the successive experiments.

Oocyte activation and embryo culture

In vitro matured oocytes were washed three times with NCSU-23 medium and activation medium. For electrical activation, oocytes were transferred between electrodes covered by activation medium in a chamber connected to an electrical pulsing machine (Cyto-pause 4000, Cyto Pulse Science Ltd., USA). The method of electrical stimulation was dependent on the experiment. In study 1, oocytes were activated by one 30-μsec pulse of 1.0, 1.5, 2.2 or 2.7 kV/cm DC. In study 2, oocytes were activated by one 30-μsec or 60-μsec pulse of 2.2 kV/cm DC. In study 3, oocytes were activated by 1 or 3 consecutive 30-μsec pulses of 2.2 kV/cm DC. Electrically activated oocytes were transferred into embryo culture medium supplemented with 7.5 μg/ml cytochalasin B (CB) for 3 h at 38.5°C in a humidified atmosphere of 5% CO_2. In study 4, oocytes were treated with 10 μM Ionomycin for 5 min followed by 2 mM 6-DMAP for 3-4 h. For embryo culture, embryos were washed 3 times in embryo culture medium, and placed in 25 μl microdrops of embryo culture medium under mineral oil and cultured at 38.5°C in a humidified atmosphere of 5% CO_2. In study 5, embryo culture medium was G1.3/G2.3 (supplemented HSA, Vitrolife Kungsbacka and Sweden), NCSU-23 and PZM-3. In study 6, embryos were cultured by an optimized protocol established in the previous studies using PZM-3 supplemented with or without 5 μg/ml insulin. Cleavage and blastocysts were checked on day 2 (Figure 1A) and on day 7 (Figure 1B and C), respectively. Blastocysts were stained with Hoechst 33342 to count the number of nuclei (Figure 1D).

Preparation of MEF feeder

Isolation, culture and passage of MEF were prepared as

Table 1. Effect of field strength on the electro-activated oocytes*

Field strength	No. of oocytes	No. of lysed oocytes (%)	No. of cleaved embryos (%)	No. of blastocysts (%)
1.0/30×1	66	0 (0.0)	37 (56.1)^{a}	3 (4.6)^{d}
1.5/30×1	95	0 (0.0)	67 (70.5)^{ab}	12 (12.6)^{e}
2.2/30×1	107	0 (0.0)	81 (75.7)^{bc}	19 (17.8)^{e}
2.7/30×1	58	4 (6.9)	44 (75.9)^{bc}	7 (12.1)^{e}

* Values in the same row with different superscripts are significantly different ($p<0.05$). The following Tables are the same.

Table 2. Effect of pulse duration on the activation of porcine oocytes

Protocol of electrical activation	No. of oocytes	No. of lysed oocytes (%)	No. of cleaved embryos (%)	No. of blastocysts (%)
2.2/30×1	92	1 (1.1)	70 (76.1)	19 (20.7)
2.2/60×1	74	6 (8.1)	56 (75.7)	10 (13.5)

described previously (Robertson et al., 1987). The MEF within passage 5 were inactivated by the treatment with DMEM medium containing 10% NBS and 10 μg/ml mitomycin C for 2-3 h at 37°C, in a humidified atmosphere of 5% CO_2, and were plated at a density of 1.2×10^5 cell per well with coated 0.1% gelatin in four-well dishes.

Isolation of porcine parthenogenetic embryonic stem cells

The 7th day porcine parthenogenetic blastocysts (pPBs) were directly cultured on MEF feeder layers (Method A); the other group (Method B) were lysed by 0.2% pronase for 2-3 min, then the pPBs were transferred to MEF feeder layers. The embryos were maintained at 38.5°C in 5% CO_2. The medium of ESCs consisted of 5% FCS (ES Cell-Qualified; Invitrogen Corp, Carlsbad, CA, USA), 15% KSR (Knockout serum replacement, Invitrogen Corp., Carlsbad, CA, USA), 0.1 mM 2-mercaptoethanol, 2 mM glutamine (Gibco), 0.1 mM non-essential amino(Gibco), 100 IU/ml penicillin, 100 IU/ml streptomycin, 5 ng/ml LIF(Chemicon Interational Inc.,Temecula,CA,USA) and 10 ng/ml bFGF in Dulbecco's modified Eagle's medium(DMEM). The medium was changed every 48 h. The ICM colonies were formed at 3-7 day after plating and were subcultured until the typical colonies were formed. Then these typical colonies were carefully picked up by a finely drawn pipette, dissociated into small clumps, and seeded onto new plates containing MEF feeder layers in ESCs culture medium.

Identification of porcine parthenogenetic embryonic stem cells

Alkaline phosphatase (AP) activity was determined essentially as described by Moore and Piedrahita (Moore et al., 1996). Briefly, culture plates were rinsed three times with Ca^{2+}- and Mg^{2+}- free PBS (PBS-) and fixed in 4% formaldehyde in PBS- for 10-15 min at room temperature. Fixed cells were washed three times with PBS- and stained in paphtol AS-MX phosphate (200 μg/ml) and Fast Red TR salt (1 mg/ml) in 100 mM tris buffer (pH 8.2) for 10-30 min at room temperature. Staining was terminated by washing cultures in PBS- to evaluate the characteristic colonies and count AP positive colonies.

Statistical analysis

At least three replicate trials were conducted for each experiment. Oocytes were randomly distributed in each experimental group. Cell numbers in blastocysts were presented as mean±SD. Results were analysed using Pearson's Chi-square analysis, with $p<0.05$ set as the level of statistical significance.

RESULTS

Effect of field strength on the electro-activated oocytes

These experiments were carried out to optimize field strength on electro- activated oocytes. As the electric field strength increased from 1.0 to 2.7 kV/cm, cleavage rate of parthenogenetic embryos increased gradually. The oocyte lysis rate was significantly increased at 2.7 kV/cm field strength. Rates of cleavage in the 2.2 and 2.7 kV/cm groups were significantly increased as compared to the 1.0 kV/cm group. No differences in rate of blastocyst formation were observed among the 1.5, 2.2 and 2.7 kV/cm groups. However, more blastocysts developed from embryos among these three groups than from embryos in the 1.0 kV/cm group ($p<0.05$) (Table 1).

Effect of pulse duration on the activation of porcine oocytes

As shown in Table 2, compared with 60-μsec pulse duration, the rate of blastocysts was increased and the rate of oocytes lysis was decreased when the field strength was 2.2 kV/cm and duration was 30-μsec, but there was no significant difference ($p>0.05$).

Effects of number of electrical pulses on the activation of porcine oocytes

The number of consecutive pulses (1 or 3) was examined in oocytes activated at 42-44 h post maturation, with pulse duration set at 30-μsec and voltage field strength

Table 3. Effect of number of electrical pulses on the activation of porcine oocytes

No. of electrical pulses	No. of oocytes	No. of lysed oocytes (%)	No. of cleaved embryos (%)	No. of blastocysts (%)
1	137	0 (0.0)	113 (82.5)	41 (29.9)[a]
3	112	6 (5.4)	81 (72.3)	9 (8.0)[b]

Table 4. Effect of different method of activation on the developmental ability of porcine oocytes

Method of activation	No. of oocytes	No. of cleaved embryos (%)	No. of blastocysts (%)
Electro-activated	124	98 (79.0)[a]	24 (19.4)[c]
Ionomycin/6-DMAP	87	52 (59.8)[b]	3 (3.4)[d]

Table 5. Effect of different embryo culture media on the developmental ability of porcine oocytes

Embryo cultured media	No. of oocytes	No. of cleaved embryos (%)	No. of blastocysts (%)	Cell no. of blastocysts (%)
G1.3/G2.3	94	67 (71.3)	14 (14.9)	35.8±7.8 (n = 14)
NCSU-23	112	92 (82.1)	23 (20.5)	29.6±9.7 (n = 17)
PZM-3	107	90 (84.1)	27 (25.2)	36.1±10.2 (n = 21)

Table 6. Effect of insulin in PZM-3 media on the development of parthenogenetically activated embryos

Insulin	No. of oocytes	No. of cleaved embryos (%)	No. of blastocysts (%)	No. total cell of blastocysts
(+)	105	95 (90.5)	39 (37.1)	44.3±9.1[a] (n = 17)
(-)	89	74 (83.1)	26 (29.2)	33.9±11.7[b] (n = 13)

Table 7. Effect of isolation method on the attaching rate of pPB and the formation of ICM colony

Isolation method	No. of blastocyst	Attaching rate/%	ICM colony forming rate/%
Method A*	16	12.5 (2/16)[a]	50.0 (1/2)[c]
Method B	16	56.3 (9/16)[b]	88.9 (8/9)[d]

* Method A: pPBs were directly cultured on MEF feeder layers.
Method B: pPBs were lysed by 0.2% pronase for 2-3 min to remove the ZP, then were transferred to feeder layers.

for 2.2 kV/cm. As shown in Table 3, the blastocyst rate which resulted from a single pulse (29.9%) was significantly higher than that of the other group (8.0%) from 3 pulses ($p<0.05$).

Effects of different method of activation on the developmental ability of porcine oocytes

As shown in Table 4, for porcine oocyte parthenogenetic activation methods, the rates of cleavage and blastocyst formation were significantly increased in electrical activation as compared to chemical activation with ionomycin/6-DMAP($p<0.05$).

Effects of different embryo culture medium on the developmental ability of porcine oocytes

The rate of cleavage and blastocyst formation in NCSU-23 and PZM-3 embryo culturing media were higher than those in G1.3/G2.3 serial cultured media, but there were no significant differences among the three groups (Table 5). The total cell number of blastocysts showed no significant differences among culture media ($p>0.05$).

Effect of insulin on the development of parthenogenetically activated embryos

As shown in Table 6, the total cell number in PZM-3 embryo culture media containing insulin was significantly higher than that of the control (no insulin) ($p<0.05$). The rates of cleavage and blastocyst formation in the group containing insulin were higher than those of the control (no insulin), but there was no significant difference between the two groups ($p>0.05$).

Effect of isolation method on the attaching rate of pPB and the formation of ICM colony

32 expanded pPB in day 7 were cultured in Method A (intact embryo culture) and Method B (nude embryo culture). It was very difficult for pPB to attach to feeder layers in Method A and only two of them attached. The rates of pPB attachment to feeder layers(Figure 2A) and ICM colony formation (Figure 2B and C) were 56.3% and 88.9%, respectively, in Method B which were significantly greater than those in Method A ($p<0.05$) (Table 7). ESCs-like colonies were positive for AP staining (Figure 2D).

DISUSSION

Protocol of activation

At present, there are many different protocols of electrical activation reported from different laboratories containing different sources of porcine oocytes, quality of porcine oocytes, maturation degrees, seasons of experiment, maturation culture media, equipment and media of electro-fusion (Liu et al., 1997; Grupen et al., 2002; Zhu et al., 2002; Fan et al., 2003; Lee et al., 2004; Jang et al., 2005;Yi

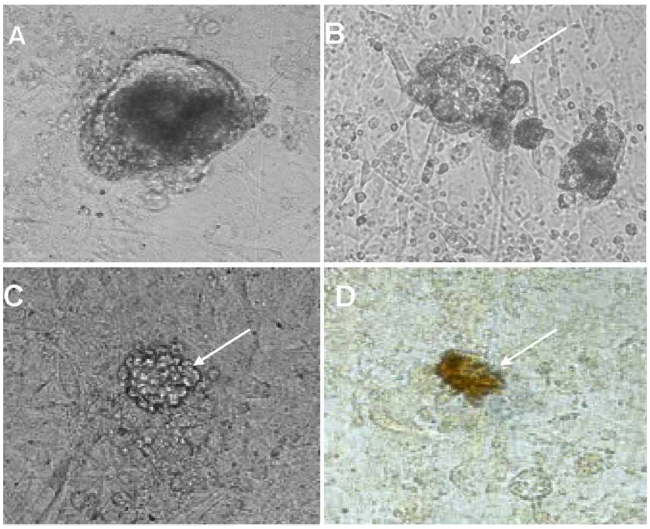

Figure 2. The growth behaviors of pPBs and the formation of porcine primary PESCs-like colonies on MEF feeder. A: pPBs were attached onto MEF feeder at the 2nd day after culturing, ×200; B-C: Primary porcine PESCs-like colony ×100; D: Colony of primary porcine PESCs-like, positive for AP staining ×100 (Arrow is referred to porcine PESCs-like colony).

et al., 2005; Somfai et al., 2006; Lee et al., 2007). Our data indicated that the ratio of cleaved embryos increased following field intensity enhanced within a certain degree, which was consistent with other studies (Zhu et al., 2002; Lee et al., 2004). Multiple pulses of electrical stimulation proved to be beneficial to oocyte activation and subsequent embryonic development after parthenogenetic activation (Vitullo et al., 1992; Collas et al., 1993; Grupen et al., 1999). Our experiment suggested that a single pulse of electrical stimulation was sufficient to activate pig oocytes, which confirmed the notion of Lee et al. (Lee et al., 2004). Better ratios of cleaved embryos (90.5%) and blastocysts (37.1%) were obtained using 2.2/30×1 to electrically activate porcine oocytes in our experiments.

Chemical activation with ionomycin/6-DMAP improved the developmental ability of bovine oocytes (Yamazaki et al., 2005; Hwang et al., 2006). Most successful somatic cell cloned pigs were reported using electrical activation methods (Onishi et al., 2000; Lee et al., 2005). Hyun (Hyun et al., 2003) holds the view that a single electrical pulse could produce a high development ratio of cloned embryos and chemical activation, such as ionomycin/6-DMAP, was not necessary. However, Betthauser (Bettauser et al., 2000) obtained a better ratio of blastocysts, pregnancy and total cell number of blastocysts using ionomycin/6-DMAP activation. The results of this work demonstrated that, compared with ionomycin/6-DMAP activation, 2.2/30×1 electrical activation significantly increased the development ratio of parthenogenetically activated embryos.

Culture of porcine parthenogenetic embryos

In vitro culture systems for porcine embryos are relatively inefficient compared with other domestic species (Swain et al., 2001). Culture conditions have apparently contributed to low developmental rate (Bettauser et al., 2000; Prather et al., 2000). NCSU-23 is one of the most successful media for porcine embryo culture *in vitro* (Onishi et al., 2000; Lee et al., 2005). However, it was reported that PZM-3, based on the composition of pig oviductal fluid with supplementary amino acids, supported more development to the blastocyst stage than NCSU-23 (Yoshioka et al., 2002). G1.3/G2.3 serial culture medium is designed commercially for human assisted reproduction (Gardner et al., 1998), but has been shown to support development of bovine (Krisher et al., 1993) and porcine (Ghandi et al., 2001; Swain et al., 2001) embryos as well. Our experiments found that the cleavage rate and blastocyst rate of porcine parthenogenetic embryos in G1.3/G2.3 culture medium were lower than those in NCSU-23 (consisting of 4 mg/ml BSA) and PZM-3 media. The results suggested that G1.3/G2.3 serial culture medium was not as effective as NCSU-23 and PZM-3 media in supporting pPBs development. The basic components of NCSU-23 and PZM-3 are alike, but PZM-3 includes essential and non-essential amino acids. The addition of certain amino acids was beneficial to porcine embryo development (Koo et al., 1997). Amino acids may relieve some of the stress inherent in the *in vitro* environment at early cleavage stages, thereby allowing a better quality embryo to develop (Swain et al., 2001). This result was consistent with the report of Im et al. (Im et al., 2004). When added to the culture medium, insulin improved bovine blastocyst development (Stefanello et al., 2006). Our results showed that insulin significantly increased the total cell number of blastocysts, which confirmed the notion that insulin played an important role in embryo development by decreasing apoptosis and increasing cell proliferation (Augustin et al., 2003).

Isolation of porcine parthenogenetic embryonic stem cells

Mouse (Allen et al., 1994), bovine (Wang et al., 2005) and nonhuman primate (Cibelli et al., 2002; Vrana et al., 2003) PESCs derived PBs have been obtained in recent years, but there is no report on the isolation of porcine PESCs. Establishment of PESCs lines is not only a good model for human PESCs, but also can be used for cell-based therapy of diseases (diabetes) and research on imprinting gene expression and function.

The attachment and colony formation rates of pPBs in method B were clearly higher than those in method A, which indicated that the removal of zona pallucida (ZP) were beneficial for the plating of pPBs and ESCs-like colony formation. The total cell number of blastocysts derived from nuclear transfer, *in vitro* fertilization and parthenogenesis were 66, 66 and 49 respectively, which were significantly less than those from *in vivo* blastocysts (about 200) (Bettauser et al., 2000). This result could possibly be due to the difficulty of plating pPBs and, in addition, the cavity of expanded blastocysts is too big to plate because of floating. Therefore, the process of isolation of porcine PESCs from ICM could be feasible. Porcine PESCs-like colonies swelled and compacted on MEF feeder layers, the morphology of which was similar to that of mouse ESCs.

IMPLICATIONS

We investigated establishment of a stable system for obtaining pPBs *in vitro*, and isolated porcine PESCs colonies derived from pPBs which have the characteristics of ESCs. However, much work is still needed to explore the pluripotency of porcine PESCs.

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