Original Article

Establishment of Porcine Embryonic Stem-like Cells from Parthenogenetic and *in vivo* Derived Embryos

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Abstract

The study was conducted to examine the effect of different parthenogenetic activation (PA) protocols on developmental competence and quality of embryos, and to establish the parthenogenetic embryonic stem (ptES)-like cells in pigs. Compacted cumulus-oocyte complexes (COCs) were collected from porcine ovaries and then cultured in M199-medium. After 44 hrs of culture, matured MII-oocytes were selected and divided into three groups. Oocytes in each group were stimulated with three different activation protocols as protocol I: 1.36 kV/cm, 30 µsec, 2 pulses; protocol II: 1.50 kV/cm, 60 µsec, 2 pulses and protocol III: 1.0 kV/cm, 80 µsec, 3 pulses, and followed by exposure to 6-dimethylaminopurine (6-DMAP) for 4 hrs. The development to blastocyst stage at day 7 was significantly greater in protocol III-activated oocytes (31.88%) than in protocol I- and II-activated oocytes (2.83% and 25.88%, respectively; $p<0.05$), however, no significant difference in the mean number of blastocyst cells among the groups. Subsequently, whole ZP-free blastocysts produced from protocol III were cultured on STO feeder layers to evaluate the establishment of ES cells by comparing them with those produced *in vivo*. No forming of primary ES-like colonies was found in PA group comparing with *in vivo* group (0% and 25%, respectively; $p<0.05$). The primary colonies derived from *in vivo* blastocysts showed typical morphology of ES cells, meanwhile revealed positive AP activity. In conclusion, our study indicates that pig ES-like cells can be established from *in vivo* produced blastocysts and their blastocysts formation was optimal when three 80-msec consecutive pulses of 1.0 kV/cm was used, however, the high quality of blastocysts in term of the number of ICMs is a crucial criterion to lead the success of ptES-like cells establishments.

Keywords: Embryonic stem-like cells, embryos quality, parthenogenetic activation, pig

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บทคัดย่อ

การสร้างเซลล์ที่มีลักษณะคล้ายเซลล์ต้นกำเนิดตัวอ่อนสุกรจากตัวอ่อนที่ผลิตโดยวิธีการกระตุ้นด้วยกระแสไฟฟ้าและจากการปฏิสนธิภายในร่างกาย

ศศิธร พนโสภณกุล[1, 2] ธีรวัฒน์ ธาราศานิต[1] มงคล เตชะกำพุ[1]*

ศึกษาผลของวิธีการกระตุ้นโอโอไซต์ด้วยกระแสไฟฟ้าในขนาดที่แตกต่างกันต่อความสามารถในการพัฒนาและคุณภาพตัวอ่อนที่ได้และสร้างเซลล์ที่มีลักษณะคล้ายเซลล์ต้นกำเนิดจากตัวอ่อนสุกรที่เจริญมาจากการกระตุ้นด้วยกระแสไฟฟ้า ทำการเก็บโอโอไซต์ที่ถูกหุ้มล้อมด้วยกลุ่มเซลล์คูมูลัส (COCs) จากรังไข่สุกรและเลี้ยงในน้ำยาเลี้ยงชนิด M199 นาน 44 ชม. คัดเลือกและแบ่งโอโอไซต์ระยะเมตาเฟส (M II) ที่เจริญเต็มที่ออกเป็น 3 กลุ่ม โดยโอโอไซต์แต่ละกลุ่มจะถูกกระตุ้นด้วยกระแสไฟฟ้าที่แตกต่างกัน 3 วิธี คือ วิธีที่ 1 กระตุ้นด้วยกระแสไฟฟ้าขนาด 1.36 kV/cm นาน 30 μsec จำนวน 2 ครั้ง วิธีที่ 2 กระตุ้นด้วยกระแสไฟฟ้าขนาด 1.50 kV/cm นาน 60 μsec จำนวน 2 ครั้ง และวิธีที่ 3 กระตุ้นด้วยกระแสไฟฟ้าขนาด 1.0 kV/cm นาน 80 μsec จำนวน 3 ครั้ง เลี้ยงโอโอไซต์ในน้ำยาเลี้ยง (NCSU-23) ที่เสริมด้วยสาร 6-DMAP นาน 4 ชม. ภายหลังทำการเลี้ยงตัวอ่อนนาน 7 วัน พบว่าโอโอไซต์ที่ถูกกระตุ้นด้วยวิธีที่ 3 มีอัตราการเจริญของตัวอ่อนระยะ บลาสโตซิสมากกว่าโอโอไซต์ที่ถูกกระตุ้นด้วยวิธีที่ 1 และ 2 อย่างมีนัยสำคัญ ($p<0.05$) แต่ไม่พบว่ามีความแตกต่างของค่าเฉลี่ยจำนวนเซลล์ของตัวอ่อนในแต่ละกลุ่มทดลอง เมื่อนำตัวอ่อนระยะ บลาสโตซิสที่ผลิตได้จากการกระตุ้นด้วยวิธีที่ 3 และไม่มีส่วนของเปลือกหุ้มตัวอ่อนมาทำการเลี้ยงลงบนเซลล์พี่เลี้ยงชนิด STO เพื่อประเมินการสร้างเซลล์ต้นกำเนิดตัวอ่อนโดยเปรียบเทียบกับบลาสโตซีสที่ผลิตได้จากการปฏิสนธิภายในร่างกาย (*in vivo*) พบว่ามีการเจริญของกลุ่มเซลล์แรกเริ่มที่มีลักษณะคล้ายเซลล์ต้น กำเนิดตัวอ่อนเฉพาะในกลุ่มของตัวอ่อน *in vivo* ($p< 0.05$) กลุ่มเซลล์ดังกล่าวแสดงรูปร่างเหมือนเซลล์ต้นกำเนิดตัวอ่อน และให้ผลบวกเมื่อทำปฏิกิริยากับอัลคาไลน์ฟอสฟาเตส (AP) สรุปได้ว่าสามารถสร้างเซลล์ที่มีลักษณะคล้ายเซลล์ต้นกำเนิดตัวอ่อนสุกรได้และการกระตุ้นโอโอไซต์ด้วยกระแสไฟฟ้าขนาด 1.0 kV/cm นาน 80 μsec จำนวน 3 ครั้ง ให้ผลการเจริญของตัวอ่อนระยะบลาสโตซิสดีที่สุด อย่างไรก็ดีพบว่า จำนวนเซลล์ในกลุ่มมวลเซลล์ชั้นใน (ICM) ของตัวอ่อนมีผลอย่างมากต่อความสำเร็จของการสร้างเซลล์ต้นกำเนิดจากตัวอ่อนที่ผลิตจากการกระตุ้นด้วยกระแสไฟฟ้าในสุกร

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Introduction

Embryonic stem (ES) cells are pluripotent cells that possesses the capacity of virtually unlimited self-renewal for long-term propagation and broad differentiation potentials, generally known to provide several benefits for biomedical research and clinical applications (Murry and Keller, 2008). Human (h) and mouse (m) ES cell lines are the two predominant species-specific models that have been intensively investigated by researchers over the past two decades (Evan et al., 1990; Thomson et al., 1998). Currently, there are many attempts to produce ES cell lines from large mammals, such as pig, because these animals are more physiologically and immunologically similar to the human (Kararli, 1995; Schuurman and Pierson, 2008). Because of its important features, establishment of porcine ES cells has therefore become more relevant and reliable for clinical translation studies for both human and veterinary medicine when compared with mES cells. Unfortunately, none of the porcine ES cell lines reported to date has satisfied all criterions essentially required for the definition of embryonic stem cells and these cell lines are usually defined as ES-like cells.

Isolation and culture of porcine ES-like cells have frequently used *in vivo* produced blastocysts (Evan et al., 1990; Piedrahita et al., 1990[a]; Strojeck et al., 1990) as quality of these embryos is expected to be higher than those *in vitro* produced embryos. This approach is however labor-intensive and the animals need to be euthanized at the time of embryo collection. By contrast to *in vivo* produced embryos, blastocysts produced from *in vitro* using artificial activation (parthenogenesis) can serve as an alternative source of embryos to establish parthenogenetic ES cells in pigs (Brevini and

Gandolfi, 2008). Parthenogenetic embryonic stem (ptES) cells can be produced from unfertilized oocytes by artificial activation that mimics the sperm penetration by generating transient rises of intracellular free calcium (Ca^{2+}) concentration either with electrical stimulation or with chemical treatment (Macháty et al., 1999; Lee et al., 2004; Nánássy et al., 2008).

In respect of embryo quality, it has been demonstrated that the electrical stimulation protocol is an important factor in determining the quality of embryos after parthenogenetic activation. For example, multiple pulses combined with the optimal field strength and duration are beneficial to the developmental competence of activated oocytes compared with a single pulse (Barnes et al., 1993; Collas et al., 1993). To date, several parthenogenetic activation (PA) protocols have been used but it remains unclear whether or not the different protocols would affect the quality of embryos, in terms of the number of cells within the inner cell mass of blastocysts and also the efficacy of using these embryos for establishment of ptES-like cells.

Our objectives were to test the effect of different activation protocols on developmental competence and quality of embryos, and also to examine the efficiency of ptES-like cells production from parthenogenetic embryos.

Materials and Methods

Chemicals: All chemicals were purchased from Sigma-Aldrich (St. Louis, USA), unless otherwise indicated.

Collection and culture of oocytes: Porcine ovaries were collected from a local abattoir at Nakorn Pathom province and transported to the laboratory at approximately 26-30°C in 0.9%(w/v) saline solution supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin. After 2-4 hrs of transportation, cumulus oocyte complexes (COCs) were collected from antral follicles with a diameter of 3-8 mm and washed twice with pre-warmed (37°C) Hepes-buffered Tyrode-lactate solution supplemented with 0.1%(w/v) polyvinyl alcohol (TL-Hepes-PVA). The COCs were examined at a magnification x 40 using a stereomicroscope (SMZ645 Nikon, Tokyo, Japan). Only COCs with at least three uniform layers of compact cumulus cells with homogeneous cytoplasm were used in this study.

For *in vitro* maturation, groups of 30-50 COCs were cultured in 500 µl of maturation medium at 38.5°C in a humidified atmosphere with 5%CO_2 in air. Oocyte maturation medium (IVM medium) was consisted of M199 (with Earle's salts) supplemented with 3.05 mM glucose, 26.2 mM sodium bicarbonate, 0.69 mM *L*-glutamine, 0.91 mM sodium pyruvate, 0.1 mM cysteamine, 10 ng/ml epidermal growth factor (EGF), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10%(v/v) porcine follicular fluid (pFF). For the first 22 hrs of oocyte culture, IVM medium was supplemented with 10 IU/ml equine chorionic gonadotropin (Folligon®, Intervet/Schering-Plough Animal Health, Boxmeer, The Netherlands), 10 IU/ml human chorionic gonadotropin (Chorulon®, Intervet/Schering-Plough Animal Health), after which the culture was performed in an absence of eCG and hCG. Porcine follicular fluid used for in vitro maturation was collected from 3-8 mm follicles, centrifuged at 1900x g for 30 min at 4°C, filtered and stored at -20°C until use.

Oocytes activation and embryos culture: After 44 hrs of *in vitro* maturation, cumulus cells were gently removed by repeated pipetting and only metaphase II (MII) oocytes with extruded a polar body were used for parthenogenetic activation. The denuded MII oocytes were washed twice and maintained in Ca2+-free-Hepes-buffered North Carolina State University-23 (NCSU 23) containing 0.4% BSA. PA was performed by washing the oocytes three times in activation medium containing with 300 mM mannitol, 0.1 mM $CaCl_2$, 0.1 mM $MgSO_4$, 0.5 mM Hepes and 0.01 mg/ml BSA, and then aligned the oocytes between two stainless steel electrodes covered by activation medium in a chamber connected to an electrical pulsing machine (SD 9 Square Pulse Stimulator; Grass Technologies Ltd., Massachusetts, USA). After activation, the oocytes were transferred and cultured for 4 hrs in the culture medium supplemented with 2 mM 6-dimethylaminopurine (6-DMAP). The parthenote oocytes were then cultured in groups of 30-50 in 500 µl NCSU-23 supplemented with 1% non-essential amino acid (NEAA) under mineral oil for 7 days. The embryo culture was performed at 38.5°C in a humidified condition of 5%CO_2 in air. The compositions of NCSU-23 containing 0.4% BSA has essentially been described by Peters and Wells (1993).

In vivo embryo production: *In vivo* produced-embryos were obtained from the crossbred gilts (at least 100 kg body weight). Gilts were treated with a combination of 400 IU equine chorionic gonadotropin (eCG) and 200 IU human chorionic gonadotropin (hCG) (PG 600®, Intervet Int. BV, Boxmeer, The Netherlands) by intramuscular injection and were artificially inseminated twice times at the first day of estrus (Day 0 of pregnancy) and 12 hrs later with freshly diluted semen. Blastocysts were collected between day 6 and 7 of pregnancy by flushing each uterine horn with PBS containing 4%heat-inactivated FBS, 100 IU/ml penicillin, and 100µg/ml streptomycin. Morula and early blastocysts were further cultured in NCSU-23 supplemented with 1%NEAA for 1-2 days at 38.5°C in a humidified condition of 5%CO_2 in air until they develop to expanded or hatched blastocysts.

Differential staining of blastocysts: Differential staining of the inner cell mass (ICM) and the trophectoderm (TE) cells was performed as essentially described by Thouas et al. (2001) with a minor modification. Briefly, blastocysts at 7 days were first incubated with 1%(v/v) Triton X-100, 0.01% polyvinylpyrrolidone (PVP) and 100 µg/ml propidium iodide (Molecular Probes, Invitrogen, Oregon, USA) in PBS for up to 15 sec. Blastocysts

were subsequently transferred into a fixative solution of 100% ethanol supplemented with 25 µg/ml bisbenzimide (Hoechst 33342, Molecular Probes) and maintained in this fixative at 4°C for overnight. Finally, fluorescently labeled embryos were mounted onto a glass microscopic slide in a droplet of glycerol and sealed with a coverslip. The embryos were then examined for ICM and TE cell numbers using an epifluorescent microscope (BX51 Olympus, Shinjuku, Japan). The ICM was classified as a group of embryonic cells that stained with only Hoechst 33342 (blue), while TE cells were positive to both Hoechst 33342 and propidium iodide.

Preparation of feeder layer: Mouse STO cells (ATCC CRL-1503, Manassas, USA) were used as feeder layer for culturing the ES cells. They were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen) supplemented with 10%(v/v) FBS (Gibco, Grand Island, NY), 1%(v/v) *L*-glutamine (Glutamax®, Gibco), and 1% (v/v) penicillin and streptomycin (x100 Pen-strep, Gibco). To prepare feeder layer, STO cells were mitotically inactivated with 10 µg/ml mitomycin-C for 2.5 hrs in culture medium. Cells were then dissociated by trypsinization with 0.05% (w/v) trypsin-EDTA solution (Gibco). The trypsinized cells were centrifuged at 1000 rpm for 5 min. The pellet was then resuspended with culture medium and seeded at a density of 55,000 cells/cm^2 into a 1-well dish (BD FalconTM, NJ, USA) pre-coated with 0.1%(w/v) gelatin.

Isolation and culture of inner cell masses (ICMs): Zona pellucida (ZP) of blastocysts was removed by treating the embryos with 0.25%(w/v) pronase in Hepes buffered NCSU-23. The putative ES cells (ICMs) were isolated by culture whole intact ZP-free blastocysts on STO feeder layers in ES medium at 37°C under a humidified atmosphere with 5% CO2 in air. ES medium was composed of knockout-DMEM (Gibco) supplemented with a 1:1 mixture of 10% FBS (Hyclone, Logan, UT) and knockout serum replacement (KSR; Gibco), 1 mM L-glutamine, 0.1 mM b-mercaptoethanol (Gibco), 1%(v/v) NEAA, 1%(v/v) penicillin-streptomycin, 1000 IU/ml recombinant human leukemia inhibitory factor (rhLIF; Chemicon Int., Temecula, CA, USA) and 20 ng/ml recombinant human basic fibroblast growth factor (rbFGF; Chemicon).

Approximately 4-6 days of culture, ICM outgrowths were mechanically removed from TE with a capillary glass pipette and replated on a new STO feeder layer for further expansion. Expanded colonies were mechanically dissociated using mechanical transfer method as described by Oh et al. (2005). The resulting colonies were continuously passaged until being differentiated or no colony was obviously formed.

Alkaline phosphatase (ALP) staining: Alkaline phosphatase activity was used as a generalized ES cell marker in this study. To examine the ALP activity, putative ES colonies were washed with PBS containing Ca^{2+} and Mg^{2+} and fixed for 2 min with 4%(w/v) paraformaldehyde. ALP staining technique was performed using an ALP histochemistry kit (Sigma, St. Louis, USA) according to the manufacturer's instruction. The ES cell colonies were finally visualized under an inverted light microscope.

Experimental design:

Experiment I: Effect of different activation protocols on developmental competence and quality parthenogenetic embryos

After *in vitro* maturation, MII oocytes were randomly assigned to stimulate with three different activation protocols:

Protocol I: two 30-µsec direct current (DC) pulses of 1.36 kV/cm

Protocol II: two 60-µsec DC pulses of 1.5 kV/cm

Protocol III: three 80-µsec DC pulses of 1.0 kV/cm

The developmental competence in terms of cleavage and blastocyst rates was evaluated at 48, 120, 144 and 168 hrs after activation, respectively. The blastocyst quality was assessed by a presence of the cell numbers of ICM and TE.

Experiment II: Establishment of parthenogenetic embryonic stem (ptES) cells

The PA technique was selected from experiment I according to the results of the number and quality of blastocysts obtained. A total of 25 ZP-free PA blastocysts were cultured on mitomycin C-inactivated mouse STO feeder layers. The rates of blastocysts that attached and primary colony of ES-like cells were recorded. The ES-like cells were also characterized by morphological appearance and generalized pluripotent markers (alkaline phosphatase activity). *In vivo*-derived blastocysts (n=8) were used as a control group in this study.

Statistical analysis: Data as expressed by mean ± standard error of the mean (SEM) was pooled from at least 3 independent replicates. Developmental competence and quality of PA embryos among experimental groups were compared by ANOVA and protected least significant different (LSD) statistical test. The efficacy of ES cells derivation between PA- and *in vivo*-produced blastocysts was compared using the χ^2-test. Statistical analysis was carried out with SPSS version13.0 software (SPSS Inc., Chicago, IL). A value of $p<0.05$ was considered to be statistically significant.

Results

Experiment I: Effect of different activation protocols on developmental competence and quality parthenogenetic embryos

After 44 hrs of IVM, 83.6% (468/560) of oocytes exhibited the 1st polar body extrusion (MII stage). As shown in table 1, oocytes activated with protocol II and III had a significant higher rate of cleaved embryos than those activated with protocol I (77.2% and 82.4% vs 34.5%, respectively; $p< 0.05$). PA of protocol III gave the highest blastocyst rate of 28.9% and 29.9% on day 6 and 7 of culture,

respectively. These values were also significantly higher than those obtained from protocol I (6.9% in both periods) and protocol II (21.4% and 22.5%; $p<0.05$). Although there was no statistical difference in the mean number of blastocyst cells ($p>0.05$) among protocols, the embryos activated by protocol II and protocol III appeared to have a higher ICM cells number than those activated by protocol I (Table 2).

Table 1 Effect of different activation protocols on *in vitro* development of porcine parthenogenetic embryo.

Treatment	No. of activated oocytes	No. of cleaved embryos (%mean±SEM)	No. of blastocysts (%mean±SEM)		
			Day 5	Day 6	Day 7
Protocol I	167	56(34.5±2.9)[a]	7(4.3±0.7)[a]	11(6.9±1.0)[a]	11(6.9±1.0)[a]
Protocol II	147	115(77.2±4.8)[b]	20(13.5±2.8)[b]	32(21.4±2.8)[b]	34(22.5±3.1)[b]
Protocol III	154	128(82.4±4.9)[b]	30(18.3±3.8)[b]	45(28.9±2.1)[c]	46(29.9±1.5)[c]

Protocol I: 1.36 kV/cm, 30 µsec, 2 pulses; Protocol II: 1.5 kV/cm, 60 µsec, 2 pulses; Protocol III: 1.0 kV/cm, 80 µsec, 3 pulses
[a, b, c] within a column, different superscripts denote values that differ significantly ($p<0.05$).

Table 2 Effect of different oocyte activation protocols on blastocyst quality

Treatment	No. of blastocysts	No. of cells (mean±SEM)			ICM : Total (%mean±SEM)
		ICM	TE	Total	
Protocol I	**11**	9.0±1.2	41.3±4.4	50.3±5.2	18.0±1.9
Protocol II	**23**	9.0+0.5	42.0±2.2	51.0+2.5	17.8±0.6
Protocol III	**22**	9.3±0.5	40.0±2.0	49.3±2.2	19.1±1.1

Protocol I: 1.36 kV/cm, 30 µsec, 2 pulses; Protocol II: 1.5 kV/cm, 60 µsec, 2 pulses; Protocol III: 1.0 kV/cm, 80 µsec, 3 pulses; ICM: inner cell mass and TE: trophectoderm

Experiment II: Establishment of parthenogenetic embryonic stem (ptES)-like cells

Blastocyst stage embryos from PA activation and *in vivo* produced differed in embryonic appearance. A group of ICM within the blastocoels was clearly observed in *in vivo* blastocysts compared to those PA embryos (Figure 1). These embryos adhered onto feeder layers approximately 1-2 days after initiation of culture and the attachment rate was not significantly different between PA and *in vivo* derived embryos (Table 3). The trophoblastic cells were then started to reexpand by 24 hrs after embryo attachment. Two of 8 (25%) *in vivo*- and 0 of 25 PA-embryos produced ES-like colonies. The two ES-like colonies were mechanically dissociated and replated on a new feeder layers, new colonies were then attached and grown-up by 1 and 2 days of subculture, respectively. All colonies demonstrated a typical morphology of ES cells, such as densely-packed mounds, containing small rounded cells and well delineated boundaries, and expressed AP activity (Figure 2). Only five passages can be maintained from the colonies and later differentiated into either epithelial-like cells or neuronal rosette formation.

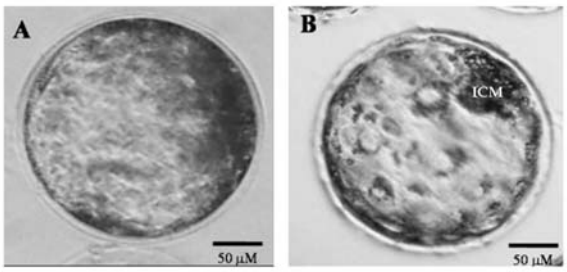

Figure 1 Morphology of day 7 blastocysts derived from parthenogenetic activation (A); and *in vivo* fertilization (B). Scale bars = 50 µM.

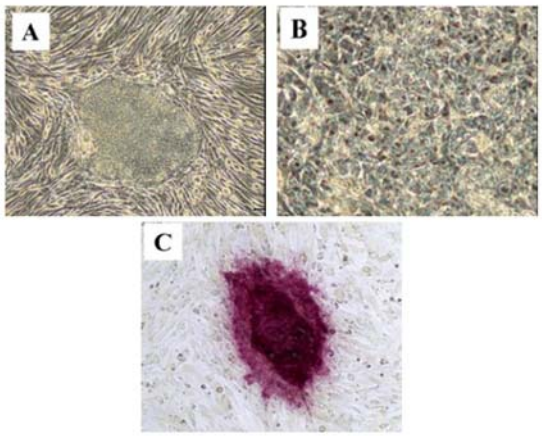

Figure 2 Porcine ES-like cells derived from day 7 of *in vivo* blastocyst, grown on STO feeder layer. A) Porcine ES-like colony at passage 2. Note the colony is densely packed with mound (x100); B) high magnification (x400) of porcine ES-like cells showing the typical ES cell morphology containing high nucleus:cytoplasm ratio, presence of nucleoli, and spaces between cells; C) Expression of positive AP activity on ES-like colony at passage 3. (x100).

Table 3 Derivation of ES-like colonies isolated from parthenogenetic (PA) and *in vivo* blastocysts (IVV).

Group	No. of blastocysts	No. of attached blastocysts (%)	No. of primary ES-like colonies (%)*
PA	25	18(72)[a]	0(0)[a]
IVV	8	8(100)[a]	2(25)[b]

*Percentage in relation to the numbers of attached blastocysts
[a,b] Within a column, values with different superscripts differ significantly ($p<0.05$)

Discussion

This study demonstrated that porcine embryos can be produced *in vitro* by means of PA as alternative source of embryo for producing ES cells as it has been reported in a number of domestic species, such as mouse (Kaufman et al., 1983), non-human primate (Cibelli et al., 2002) and human (Brevini et al., 2006). The use of these parthenogenetic embryos for producing ES cell line however depends on many factors such as stage of development (Lee et al., 2004) and also embryo quality (reviewed by Vackova et al., 2007). To date, several PA protocols have been used to produce porcine embryos *in vitro* and many of these protocols could produce high developmental rates of embryos. However, examination of embryo quality in terms of ability to use these embryos for establishment of ES cell has been restricted. In the present study, we compared the efficiency of different activation protocols on the developmental competence and quality of embryos using SD 9 pulse stimulator machine. Electrical pulses can induce transient formation of membrane pores that allow the exchange of extra- and intra-cellular calcium (Zimmermann and Vienken, 1982), a process by which mimics the fertilization process. It was found that the oocytes having been activated by the lowest field strength of 1.0 kV/cm with 3 pulses at 80 µsec (protocol III) gave the highest potential of embryos development when compared to those by 1.36 kV/cm, 30 µsec, 2 pulses (protocol I) and 1.5 kV/cm, 60 µsec, 2 pulses (protocol II). This activation protocol gave a high rate of embryo development (30% blastocyst rate) similar to previous report by Zhu et al. (2002) who demonstrated that the developmental rate of PA blastocysts was optimal when three 80 µsec consecutive DC pulses of 1.0 kV/cm were used. However, a large number of oocytes underwent degeneration when a single pulse of higher field strength (1.5 kV/cm DC) was applied (Kure-bayashi et al., 2000). It is likely that too low field strength (less than 1.2 kV/cm) and low pulse numbers are insufficient to increase calcium influx to the level needed for oocyte activation (Collas et al., 1993). In contrast, multiple pulses of high field strength (more than 1.2 kV/cm) have also been to be detrimental to the oocyte development (Lee et al., 2004). It is therefore obviously demonstrated that there is an interaction between pulse numbers and duration that affects the developmental competence of electrical activated oocytes (Prather et al., 1989; Ozil and Huneau, 2001; King et al., 2002). In the present study, we found that activation by three pulses at 80 µsec (protocol III) increased more blastocyst formation rate than those activated by two pulses either at 30 or 60 µsec (protocol I and II, respectively). It could be due to the use of field strengths less than 1.2 kV cause to be less damaging to the oocyte plasma membrane and a combination of longer pulse duration and lower field strength could increase the influx of Ca^{2+} similar to that occurs during the *in vivo* fertilization. We did not observe the difference of embryo quality in terms of the numbers of trophoblastic and ICM cells among the three different PA protocols. It is likely that the different quality of parthenogenetic pig embryos is more influenced by culture medium used for embryo culture rather than the electrical stimulus protocol (Nánássy et al., 2008).

The success of ES cell establishment was found when using in vivo blastocyst as source, at least 5 passages can be maintained. The primary colonies derived from *in vivo* blastocysts showed typical morphology of ES cells, meanwhile revealed positive AP activity Nevertheless, with the same culture condition, the primary ES-like colony from the parthenogenetic blastocyst was not formed. As the numbers of cells in ICM of blastocyst is a primary factor affecting the success of primary ES-like colony isolation, this failure of primary ES-like colony outgrowth may cause by the quality of embryos per se since the quality of embryo, in terms of trophoblastic and ICM cells, is markedly different between activation protocols (Zue et al., 2002; Cong et al., 2008) and also between *in vitro* and *in vivo* derived porcine embryos. For example, total cell number of *in vivo*-derived blastocysts is reported to be nearly 57 and 93 at day 6 (Yoshioka et al., 2002) and day 7 (Fuente & King, 1997) of development, respectively while *in vitro*-derived blastocysts have less than 55 cells at day 7 (Grupen et al., 2002; Krylov et al., 2005). Relative recently, parthenogenetic ES-like cell lines have been produced using an optimum culture system and good quality of parthenogenetic embryos (Brevini et al., 2005, Brevini et al., 2007, Kim et al., 2007), although the efficiency of producing these ES-like cells remain poor. Using our culture system, we however succeeded to isolate 25% of primary ES-like colonies from *in vivo* produced porcine embryos. Unfortunately, these primary ES-like cells were spontaneously differentiated beyond the 5th passages into epithelial-like cells or neuronal rosette formation. Maintaining of these cells in undifferentiated stage in vitro is one of major obstacles of establishment of ES cell line in this species, in particular that the culture system using to date may be not optimal to the pig ES cell culture, not like in human and mouse. It is worth to note that molecular pathways to maintain ES cells in undifferentiated stage have been shown to be different among species especially in mouse and human. For instance, mES cell requires leukemia inhibitory factor (LIF) to activate pluripotency factors, while hES cell does not require LIF to support their pluripotency. The LIF function essentially acts via a membrane bound of the heterodimerized leukemia inhibitory factor receptor (LIFR) and glycol-protein 130 (gp 130) receptor complex which regulate the undifferentiated state and self-renewal of mES cells through the Janus kinase non-receptor tyrosine kinase (JAK) and signal transducers and activators of transcription 3 (STAT3) signaling pathway (reviewed by Talbot and Blomberg, 2008). On the other hand, the activity of the LIFR and signal transduction pathways have been inconsistently detected in undifferentiated porcine ICMs and in 24 h cultured, undifferentiated epiblast tissue (Blomberg et al., 2008), whereas, LIFR expression has not been found in porcine ES-like cells (Vockova et al., 2007). However, high levels of this supplement prevent a loss of proliferation in epiblast outgrowth cultures, suggesting its role may aid in continued propagation of pES cells in culture.

In pig, the presence of LIF in ES culture medium did not help to maintain undifferentiated stage of ES-like cells as shown in this study and other reports (Talbot et al., 1993, Moore and Piedrahita, 1997). Furthermore, the characterization technique of pES cells has also been hampered by limited information of pluripotent markers that are essentially specific and standardized to pES cells. Characterization of pES cells therefore has been mainly performed by morphology assessment and some molecular markers such as alkaline phosphatase, Oct4, Nanog, SSEA-4, TRA1-60 and TRA 1-80. These cells have a typical characteristic of ES cell morphology as small and rounded cells that have large nucleus with one or two prominent nucleoli (Piedrahita et al., 1990b). In mouse and human, Oct 3/4 and Nanog transcription factors have been identified as reliable markers for ES cells since these two transcription factors are essential in maintaining the stemness of the ES cells (Nichols et al., 1998; Chambers et al., 2003). This Oct 3/4 mRNA however expresses throughout the preimplantation stage of porcine embryos and is not specific to only ICM of the embryos (Kirchhof et al., 2000).

In conclusion, our study indicates that an electrical stimulation can be alternatively used to produce parthenogenetic embryos and the high blastocysts formation rate was obtained when three 80-msec consecutive pulses of 1.0 kV/cm were used. We successfully established pES-like cells from *in vivo* derived porcine embryos, however, they spontaneously differentiated after the 5th passage. Further study therefore requires elucidating the networks of gene and factors that potentially regulate the pluripotency pathways of porcine embryonic stem cells and also the specific requirement of pES cells during *in vitro* culture.

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