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Relationship between Developmental Ability and Cell Number of Day 2 Porcine Embryos Produced by Parthenogenesis or Somatic Cell Nuclear Transfer*

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ABSTRACT : *In vitro* produced porcine embryos have potential application in reproductive biotechnology. However, their development potential has been very low. This study evaluated the *in vitro* developmental ability and quality of cloned and parthenogenetic porcine embryos having 2-4 cells or 5-8 cells on Day 2 of *in vitro* culture. Analysis of results showed that 2 to 4 cell embryos had higher ability to form blastocysts than 5 to 8 cell embryos ($p<0.05$). Blastocysts produced from culture of 2 to 4 cell embryos also contained higher cell numbers and had lower *BAX:BCLxL* transcript ratio than those produced from 5 to 8 cell embryos ($p<0.05$), thereby suggesting 2 to 4 cell embryos have higher development potential. Further investigation revealed that 5 to 8 cell embryos had higher incidence (100±0.0%) of blastomeric fragmentation than 2 to 4 cell embryos (15.2±5.5% for parthenogenetic and 27.7±7.1% for cloned embryos). This suggests that low development potential of 5 to 8 cell embryos was associated with blastomeric fragmentation. In conclusion, we have shown that morphological selection of embryos based on cell number on Day 2 of *in vitro* culture could offer a practical and valuable non-invasive means to select good quality porcine embryos. (**Key Words** : Blastomeric Fragmentation, Developmental Ability, Embryo Quality, Parthenogenesis, Porcine, Somatic Cell Nuclear Transfer)

INTRODUCTION

Somatic cell nuclear transfer (SCNT) has potential applications in the generation of transgenic cloned pigs for production of proteins of pharmaceutical importance and for xenotransplantation (Miyoshi and Sato, 2000). However, its efficiency has been very low with only 1-5% of reconstructed embryos becoming live offspring upon embryo transfer (Betthauser et al., 2000; Verma et al., 2000). This low success rate is assumed to be a cumulative result of losses at different stages of pregnancy due to several unknown causes including chromosomal abnormalities, low cell number and altered inner cell mass (ICM) rate of blastocysts, irregular-sized blastomeres, and cytoplasmic fragmentation (Wang et al., 1999; Hao et al., 2003; Wheeler et al., 2004).

Morphological assessment of embryos for their quality is a common practice for embryo selection prior to embryo transfer. However, morphology of *in vitro*-produced (IVP) porcine embryos differs with different IVP systems. In particular, cloned and parthenogenetic porcine embryos show higher level of fragmented and apoptotic cells that distort their appearance when viewed under microscope (Wang et al., 1999; Hao et al., 2003; Im et al., 2005; Mateusen et al., 2005). Improvement in embryo quality assessment parameters for cloned and parthenogenetic embryos may therefore, be important to help improve their production efficiency.

Evaluation of early developmental kinetics has recently emerged as an important non-invasive technique for determining the quality of *in vivo* or *in vitro* fertilized embryos. Fast-cleaving embryos show less fragmentation (Tanghe et al., 2004) and apoptosis (Lonergan et al., 1999) and have higher blastocyst rate (Vandaele et al., 2006), total

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cell number, ICM rate and fetal development (McKiernan and Bavister 1994; Racowsky et al., 2000). Thus, it is plausible that counting the cell number of embryos on a specified day of culture could be prognostic of their quality. However, to our knowledge, such study for cloned and parthenogenetic porcine embryos has been lacking in the literature.

Therefore, this study was designed to evaluate whether morphological selection of embryos based on cell number on Day 2 of *in vitro* culture (IVC) could offer a practical and valuable non-invasive means to select good quality porcine embryos produced by SCNT or parthenogenesis. Parthenogenetic embryos were chosen as the control instead of *in vitro* fertilized (IVF) embryos because we and others have earlier shown that development characteristics of parthenogenetic embryos resembled those of IVF and SCNT embryos (Kure-bayashi et al., 2000; Hong et al., 2005; Gupta et al., 2007a; Gupta et al., 2008a; b). Moreover, contrary to IVF embryos, use of parthenogenetic embryos avoids the confounding variation due to influence of male/sperm factor and polyspermy which is particularly common in porcine species (Han et al., 1999). Day 2 of IVC was specifically chosen for analysis because embryo transfer in the porcine is most commonly done at the 2 to 4 cell stage on Day 2 of *in vitro* culture (Kikuchi et al., 1999; Betthauser et al., 2000; Brussow et al., 2000; Yoshioka et al., 2002).

MATERIALS AND METHODS

All chemicals were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) unless otherwise specifically indicated.

Oocyte retrieval and *in vitro* maturation

Porcine ovaries were collected from a local abattoir and transported to the laboratory in saline maintained at 30 to 37°C. Cumulus oocyte complexes (COCs) were aspirated from follicles (3 to 6 mm diameter) using a 10 ml hypodermic syringe fitted with an 18 gauge needle. The COCs were washed three times in TL-HEPES media containing 1 mg/ml BSA (low carbonate TALP; Parrish et al., 1988) and matured in groups of 50 in 500 μl of Tissue Culture Medium 199 with Earle's salts (TCM-199; Gibco BRL, Grand Island, NY) supplemented with 25 mM $NaHCO_3$, 10% (v/v) porcine follicular fluid, 0.57 mM cysteine, 0.22 μg/ml sodium pyruvate, 25 μg/ml gentamicin sulfate, 5 μg/ml pFSH (Folltropin V; Bioniche Animal Health, Belleville, ON, Canada), 1 μg/ml estradiol-17β, and 10 ng/ml epidermal growth factor under mineral oil at 39°C in a humidified atmosphere of 5% CO_2 in air for 40 to 42 h as described earlier (Gupta et al., 2007b).

Preparation of donor cells

Donor cells for nuclear transfer were prepared as described earlier (Uhm et al., 2000a). Briefly, primary fetal fibroblasts were isolated from Landrace fetuses aged 35 d after mating. Cells were cultured on 60-mm tissue culture dishes (Falcon BD, NJ, USA) in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL). After 7 d of culture, cells were trypsinized and resuspended in DMEM supplemented with 10% (v/v) FBS. Cells were routinely maintained on 50 ml tissue culture flasks (Falcon BD) up to 2 to 7 passages and used as donor cells for nuclear transfer after attainment of confluence to synchronize their cell cycle stage to G_0/G_1.

Somatic cell nuclear transfer (SCNT)

Nuclear transfer was performed as described earlier (Uhm et al., 2000b) with partial modifications. Briefly, *in vitro* matured oocytes were stripped of cumulus cells in TL-HEPES supplemented with 0.1% (w/v) hyaluronidase and washed three times in TL-HEPES-BSA medium. Denuded oocytes were incubated for 20 min in TCM-199 medium supplemented with 10% (v/v) FBS and 7.5 μg/ml cytochalasin B (CB) and 5 μg/ml Hoechst 33342. Enucleation was performed by aspirating the first polar body and adjacent cytoplasm (approximately 30% of ooplasm) using a beveled pipette (25 μm internal diameter) in HEPES buffered TCM medium supplemented with 10% (v/v) FBS. Enucleation was confirmed by UV-assisted visualization of fluorescent metaphase plate in the aspirated cytoplasm contained within the enucleation pipette. Enucleated oocytes were subsequently reconstructed by inserting a small-sized (~15 μm in diameter), smooth bordered fibroblast cell into the perivitelline space of each enucleated oocyte using the same pipette as used for enucleation. Reconstructed oocytes were placed in TCM-199 medium supplemented with 10% (v/v) FBS for 30 min to allow recovery. Fusion was subsequently achieved at room temperature in fusion solution (0.3 M mannitol, 1.0 mM $CaCl_2$ and 0.1 mM $MgSO_4$) by a single DC pulse of 2.1 kV/cm for 30 μs delivered by a BTX Electro Cell Manipulator 2001 (BTX, San Diego, CA).

Parthenogenetic activation of oocytes

Parthenogenetic activation of oocytes was achieved by electroactivation as described earlier (Gupta et al., 2007a). Briefly, oocytes were washed three times in TL-HEPES-BSA medium and transferred into activation medium that consisted of 0.3 M mannitol, 0.1 mM $MgSO_4$, and 1.0 mM $CaCl_2$. Activation was then induced with a single DC pulse of 1.36 kV/cm for 30 μs. Following activation, activated oocytes were cultured in NCSU23 medium supplemented

Table 1. Primer sequences used in real time quantitative reverse transcriptase polymerase chain reaction

Gene name	Primers	Sequence (5'→ 3')	GenBank accession number	Amplicon size (bp)
BCL-XL	Forward	ACTGTGCGTGGAGAGCGTAG	AF216205	255
	Reverse	CATTTCCGACTGAAGAGCGA		
BAX	Forward	AGCGCATTGGAGATGAACTG	AJ606301	253
	Reverse	AGCCGATCTCGAAGGAAGTC		
GADPH	Forward	GGGCATGAACCATGAGAAGT	AF017079	230
	Reverse	AAGCAGGGATGATGTTCTGG		

with 7.5 μg/ml CB for 4 h before being transferred to *in vitro* culture medium for further culture.

***In vitro* culture of embryos**

Embryos were cultured in NCSU23 medium supplemented with 0.4% (w/v) BSA for 7 d as described earlier (Gupta et al., 2008b). Cleavage rates were assessed on Day 2 and blastocyst rates on Day 7 of *in vitro* culture. At 168 h post activation, blastocysts were harvested for assessment of embryo quality by Hoechst 33342 staining for counting total cell number, differential staining for ICM rate, and real time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis for *BAY*:*BCL-xL* transcripts.

Fluorescence staining for assessment of blastomeric fragmentation

For assessment of blastomeric fragmentation, 2 to 4 cell and 5 to 8 cell embryos on Day 2 of *in vitro* culture were separately stained with Hoechst 33342 and embryos having no nucleus in one or more blastomere were identified as fragmented (Wang et al., 1999). For counting the total cell number per blastocyst, Day 7 embryos were stained with Hoechst 33342. The fluorescent staining was performed as described earlier (Uhm et al., 2007a). Briefly, embryos were fixed for 5 min in fixative solution containing 2% (v/v) formalin and 0.25% (v/v) gluteraldehyde. Fixed embryos were then mounted on clean glass slides and stained with glycerol-based Hoechst 33342 (12.5 μg/ml) solution for 10 min. Stained nuclei appeared blue when visualized under UV illumination of an epifluorescent microscope fitted with a standard blue filter.

Differential staining for analysis of inner cell mass (ICM) rates

To assess the ICM rate, Day 7 blastocysts were differentially stained for ICM and trophectoderm (TE) cells as described earlier (Uhm et al., 2007b). Briefly, blastocysts were removed of their zona pellucida by treatment with Acid Tyrode's solution and washed in TL-HEPES medium containing 0.1% PVP. Zona pellucida-free embryos were then exposed to a 1:5 dilution of rabbit anti-pig whole serum for 1 h. They were then washed three times for 5 min and exposed to 1:10 dilution of guinea pig complement containing 10 μg/ml propidium iodide and 10 μg/ml Hoechst 33342 for 1 h. After brief washing, the stained embryos were mounted on clean glass slides and examined under UV light using epifluorescent microscope. Blue and red cells were counted as cells from the ICM and TE, respectively and ICM rate was calculated as: number of ICM cells/ total number of cells X 100.

Real time quantitative reverse transcription polymerase chain reaction (Real time qRT-PCR)

To analyze the relative abundance of mRNA transcripts of *BAY* and *BCL-xL* genes, blastocysts (10 per sample) were harvested at 168 h post-activation, snap frozen in lysis buffer (0.1% Polyvinyl pyrrolidone in PBS added with 1 U/μl RNAsin; Promega, WI) and processed for real time qRT-PCR as described earlier (Gupta et al., 2007c). Briefly, messenger RNA was extracted using the Dynabeads™ mRNA Direct Kit and Magnetic Particle Concentrator (Dynal Asa, Oslo, Norway) according to the manufacturer's instructions. First strand cDNA synthesis was performed using AccuPower™ RT Premix (Bioneer, Daejeon, Korea) according to the manufacturer's protocol. The mRNAs of *BCL-xL* and *BAY* genes were then amplified by real time qRT-PCR (Chromo 4™ Continuous Fluorescence Detector, MJ Research, MA, USA) with specific primer pairs (Table 1) using DyNAmo SYBR green qPCR kit (Finnzyme, Espoo, Finland) that contained modified Tbr DNA polymerase, SYBR Green, optimized PCR buffer, 5 mM $MgCl_2$ and dNTP mix including dUTP. The housekeeping gene, *GADPH*, was used as an internal standard in all experiments. In addition, a non-template control containing all the above reaction mixture but lacking cDNA was included in each PCR run. The thermoprofile for amplification was initial denaturation at 95°C for 15 min, denaturation at 97°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s and final extension at 72°C for 5 min. 40 cycles of amplification was done. Fluorescence data were acquired and analyzed by Opticon Monitor™ software version 2.03 (MJ Research, MA, USA) to generate a melting curve for distinguishing specific amplicons from non- specific ones. The relative quantification was done by 2-ddCt method. The PCR product sizes were confirmed by submarine agarose gel electrophoresis and staining with ethidium bromide.

Table 2. *In vitro* developmental competence (Mean±SEM) of porcine embryos produced by somatic cell nuclear transfer (SCNT) or parthenogenetic activation (PA)

Groups	No. of oocytes	Cleavage rate	Blastocyst rate	Total number of cells /blastocyst	ICM rate (%)
PA	337	68.6^{a}±4.5 (232)	27.5^{a}±3.2 (93)	33.2^{a}±4.7 (29)	20.2±1.7
SCNT	256	55.3^{b}±5.3 (142)	13.2^{b}±1.8 (34)	24.9^{b}±1.8 (24)	23.1±1.7

Values within parentheses indicate the number of embryos.
Values with different superscripts (a, b) within a column differ significantly ($p<0.05$).

Experimental design

Each experiment consisted of at least four replicates in each which the oocytes were from the same collection of abattoir-derived ovaries.

Experiment 1 : The first set of experiments evaluated the *in vitro* developmental ability and quality of porcine embryos, produced by parthenogenetic activation or SCNT, to obtain a base level for comparison. To assess the *in vitro* developmental ability, cleavage and blastocyst rates were evaluated on Day 2 and Day 7 respectively and expressed on the basis of number of oocytes used for culture following activation (parthenogenesis) or fusion (SCNT). To evaluate the embryo quality, blastocysts were stained with Hoechst 33342 to count the total cell number per blastocyst. In another separate experiment, blastocysts were also analyzed for ICM rate by differential staining.

Experiment 2 : To evaluate the effect of number of cell divisions on developmental ability and quality of parthenogenetic and SCNT embryos, Day 2 embryos were morphologically classified as 2 to 4 cell or 5 to 8 cell embryos and were cultured separately. After 5 d of *in vitro* culture, blastocyst rates were evaluated based on the number of embryos used for culture and were subsequently stained with Hoechst 33342 fluorescent stain to count their total cell number. In another set of separate experiments, blastocysts were also analyzed for ICM rate by differential staining and relative abundance of transcripts for pro-apoptotic *BAX* and anti-apoptotic *BCL-xL* genes by real time qRT-PCR.

Experiment 3 : In this set of experiments, we evaluated the incidence of blastomeric fragmentation in 2 to 4 cell and 5 to 8 cell porcine embryos on Day 2 of *in vitro* culture. Porcine embryos, produced by parthenogenetic activation or SCNT, were cultured *in vitro* for 2 days. On Day 2 of *in vitro* culture, the percentage of cleaved embryos displaying two to four cells or five to eight cells were recorded and subsequently stained with Hoechst 33342 to analyze the incidence of fragmentation in these embryo groups.

Statistical analyses

Data on *in vitro* development rates and ICM rates were evaluated statistically by the Binomial Model using the General Linear Mixed Models procedure (Glimmix, Statistical Analysis System Inc., Cary, NC, USA) whereas blastocyst cell numbers were compared by ANOVA (followed by Bonferroni multiple pair-wise comparison). Differences were considered significant when $p<0.05$.

RESULTS

Experiment 1

This experiment evaluated the *in vitro* developmental ability and embryo quality of porcine embryos produced by parthenogenesis or SCNT. As shown in Table 2, the cleavage and blastocyst rates were significantly higher for parthenogenetic embryos than SCNT embryos. Parthenogenetic blastocysts also contained higher cell number than their SCNT counterparts ($p<0.05$). However, the ICM rates in parthenogenetic (20.2±1.7%) and cloned blastocysts (23.1±1.7%) did not differ.

Experiment 2

When 2 to 4 cell and 5 to 8 cell embryos were cultured separately, 2 to 4 cell embryos showed higher ($p<0.05$) blastocyst rates than those of 5 to 8 cell embryos in both

Table 3. *In vitro* developmental competence (Mean±SEM) of somatic cell nuclear transferred (SCNT) or parthenogenetically activated (PA) 2 to 4 cell and 5 to 8 cell porcine embryos cultured separately after Day 2 of *in vitro* culture

Groups		No. of embryos	Blastocyst rate	Total number of cells/blastocyst	ICM rate
PA	2 to 4 cell	342	32.6^{a}±5.1 (108)	34.4^{a}±2.2	17.3^{a}±1.9
	5 to 8 cell	141	17.9^{c}±5.9 (26)	22.3^{c}±1.6	26.2^{b}±2.9
SCNT	2 to 4 cell	208	21.8^{b}±3.5 (45)	29.4^{b}±2.2	19.5^{a}±1.9
	5 to 8 cell	139	6.5^{d}±3.1 (9)	17.7^{d}±1.5	27.6^{b}±2.9

Values within parentheses indicate the number of embryos.
Values with different superscripts (a, b) within a column differ significantly ($p<0.05$).

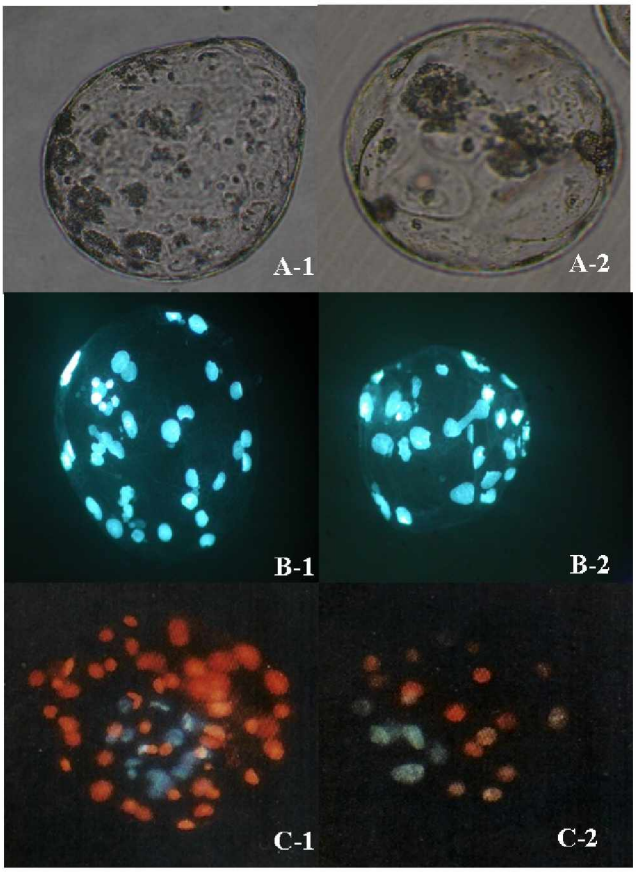

Figure 1. Porcine blastocysts produced from 2 to 4 cell (panel 1) and 5 to 8 cell (panel 2) embryos that were cultured separately from Day 2 of *in vitro* culture. A: unstained, B: after staining with Hoechst 33342, C: after differential staining (Magnification-400×).

parthenogenetic and SCNT groups (Table 3). Similarly, blastocysts from 2 to 4 cell embryos showed significantly higher cell number in both parthenogenetic and SCNT groups (Figure 1). However, the ICM rates of blastocysts from 5 to 8 cell embryos were higher ($p<0.05$) than those of 2 to 4 cell stage embryos in both parthenogenetic and SCNT groups. Real time qRT-PCR analysis of blastocysts showed that ratio of *BAY:BCL-xL* transcript abundance was 3.7 fold higher in parthenogenetic and 2.4 fold higher in cloned blastocysts derived from 5 to 8 cell stage embryos

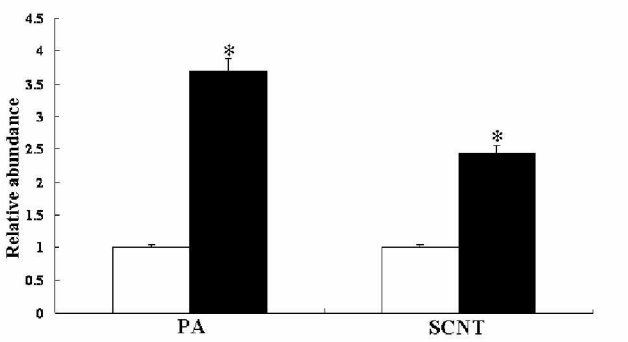

Figure 2. Relative abundance of *BAY:BCL-xL* transcript ratio in porcine blastocysts produced by somatic cell nuclear transferred (SCNT) or parthenogenetically activated (PA) 2 to 4 cell (□) and 5 to 8 cell (■) porcine embryos cultured separately after Day 2 of *in vitro* culture. GAPDH mRNA was used as internal standard. Asterisk indicates statistical difference ($p<0.05$).

compared to those derived from 2 to 4 cell stage embryos (Figure 2).

Experiment 3

To evaluate the incidence of blastomeric fragmentation on Day 2 of *in vitro* culture, parthenogenetic and cloned porcine embryos, classified as 2 to 4 cell or 5 to 8 cell embryos, were stained with Hoechst 33342 to distinguish between normal and fragmented embryos. As shown in Table 4, on Day 2 of *in vitro* culture 71.3±6.3 and 28.7±6.3% of parthenogenetic embryos and 59.2±5.8 and 40.8±5.8% of cloned embryos were at 2 to 4 cell and 5 to 8 cell stage, respectively. Interestingly, we noticed that among the apparently normal 2 to 4 cell stage embryos, 27.7±7.1% of cloned and 15.2±5.5% parthenogenetic embryos had one or more blastomeres that lacked their nucleus (Figure 3). Similarly, all apparently normal 5 to 8 cell stage cloned or parthenogenetic embryos contained one or more blastomeres that did not have a nucleus and were suggestive of fragmentation.

DISCUSSION

This study evaluated the developmental ability and quality of parthenogenetic and cloned porcine blastocysts

Table 4. Incidence of blastomeric fragmentation (Mean±SEM) in somatic cell nuclear transferred (SCNT) and parthenogenetically activated (PA) porcine embryos on Day 2 of *in vitro* culture

Group	No. of oocytes	Cleavage rate	Percentage of 2 to 4 cell stage embryos		Percentage of 5 to 8 cell stage embryos	
			Total	Fragmented	Total	Fragmented*
PA	251	68.4^{a}±3.8 (172)	71.3^{a}±6.3 (123)	15.5^{a}±2.1 (19)	28.7^{a}±6.3 (49)	100.0^{a}±0.0 (49)
SCNT	189	59.7^{b}±4.6 (113)	59.2^{b}±5.8 (67)	28.9^{b}±6.9 (19)	40.8^{b}±5.8 (46)	100.0^{a}±0.0 (46)

Values within parentheses indicate the number of embryos.

Values with different superscripts (a, b) within a column differ significantly ($p<0.05$).

* Embryos having no nucleus in one or more than one blastomere were classified fragmented.

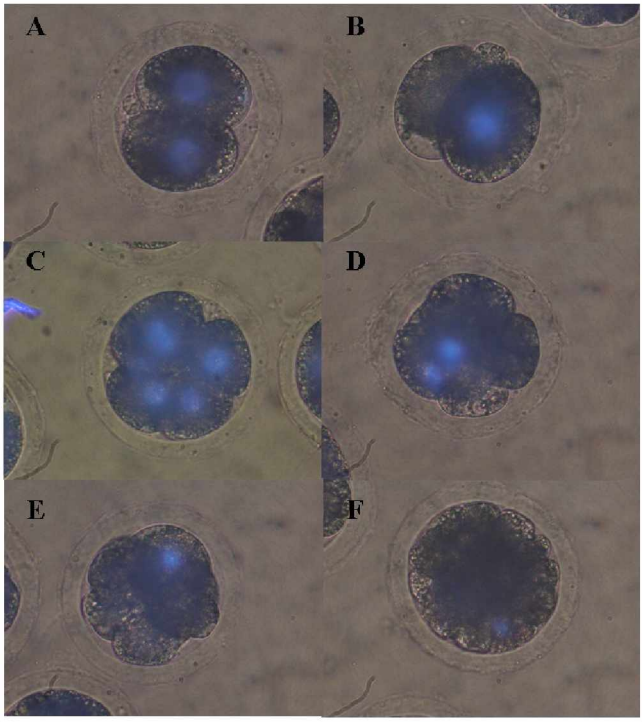

Figure 3. Porcine embryos stained with fluorescent Hoechst 33342 stain- A: normal 2-cell embryo, B: fragmented 2-cell embryos, C: normal 4-cell embryos, D: fragmented 4-cell embryo, E: fragmented 5 to 8 cell embryos, F: fragmented >8 cell embryo. (Magnification, 200×).

produced by separate culture of 2-4 cell and 5-8 cell embryos on Day 2 of *in vitro* culture. Embryo quality was assessed based on blastocyst cell number, ICM rate and expression of apoptosis-related genes that are well known indicators of embryo quality (Yoshioka et al., 2002; Jang et al., 2004; Gupta et al., 2007a; c; Kim et al., 2007; Uhm et al., 2007b; Gupta et al., 2008b; Kim and McElroy, 2008; Zhang et al., 2009). Our results show that 2-4 cell parthenogenetic and cloned porcine embryos had higher development competence than those of 5-8 cell stage embryos and hence, morphological selection of such embryos may be a useful non-invasive tool to improve cloning efficiency after embryo transfer.

Earlier it had been reported that early cleaved IVF embryos had higher development potential than those of late cleaved embryos which was suggested to be associated with their intrinsic high quality or factors such as paternal influences on the S- and G1-phase of zygotes (Eid et al., 1994; Comizzoli et al., 2000), aberrant maternal inherited cytoplasm (Liu and Keefe, 2000; Meirelles et al., 2004), gender differences (Mittwoch 1989; Yadav et al., 1993) and chromosomal abnormalities (Kawarsky et al., 1996; Viuff et al., 2001). However, we found that 5 to 8 cell PA and SCNT embryos had lower development rate to blastocyst stage, contained lower cell number per blastocyst and had higher ratio of *BAX:BCL-xL* transcripts per blastocyst than those of 2 to 4 cell embryos, thereby suggesting them to be of poor quality. The reason for this discrepancy in our study and published reports is not fully clear. It may simply be a characteristics of parthenogenetic and cloned embryos as they show higher level of blastomeric fragmentation and apoptosis than those of fertilized embryos (Wang et al., 1999; Hao et al., 2003; Im et al., 2005; Mateusen et al., 2005). Indeed, we found that a high percentage of apparently normal 2 to 4 cell as well as 5 to 8 cell embryos lacked a nucleus in one or more blastomeres. The proportion of such fragmented embryos was much higher for 5 to 8 cell stage embryos (100%) compared to 2 to 4 cell stage cloned (28.9±6.9%) or parthenogenetic (15.5±6.3%) embryos. Since the presence of fragmented blastomeres in an embryo may cause distortion of division planes, interference with normal cell-cell contact, abnormal compaction, cavitation, and blastocyst formation (Lindner and Wright 1983; Puissant et al., 1987; Mateusen et al., 2005), it may have resulted in lower development rate of 5-8 cell embryos compared to 2-4 cell embryos. Moreover, in our study, we grouped 2-4 cell and 5-8 cell stage embryos separately. Thus, it is also likely that grouping of such qualitatively better 2-4 cell stage embryos might have resulted in the prevention of apoptosis by a cooperative interaction between embryos. Conversely, lower blastocyst rate from 5-8 cell stage embryos could be due to removal of high quality embryos at day 2 from the culture droplet, which may have resulted in a decrease of the cooperative action in the droplets and hence higher apoptosis. The cooperative interaction in group culture associated with autocrine or paracrine factors secreted by the embryos themselves has been well recognized (Ferry et al., 1994; Kane et al., 1997).

ICM rate is also a valuable indicator of embryo quality (Yoshioka et al., 2002; Kim et al., 2007; Kim and McElroy, 2008). While the proportion of ICM cells in blastocysts was suggested to be crucial for later postimplantation development (Iwasaki et al., 1990), an increased ICM rate was suggested to be associated with "large offspring syndrome" in cloned animals (Barcroft et al., 1998; Leese et al., 1998). Therefore, an optimal ICM rate appears to be important. We found that blastocysts produced from 5 to 8 cell stage embryos had higher ICM rate than those produced from 2-8 cells embryos and approached those reported for *in vivo* produced porcine embryos (~30%; Yoshioka et al., 2002) thereby, suggesting them to be of better quality. However, when corrected for total nuclei number, 5-8 cell derived blastocysts had higher number of TE cells and similar number of ICM cells. Thus, low cell number and high ICM rate in blastocysts derived from 5-8 cell embryos are suggestive of their poor quality.

Embryo transfer in the porcine is most commonly done at the 2 to 4 cell stage on Day 2 of *in vitro* culture (Kikuchi et al., 1999; Betthauser et al., 2000; Brussow et al., 2000; Yoshioka et al., 2002). Since, 5-8 cell embryos on Day 2 of culture showed high fragmentation and low quality blastocyst, we propose that morphological selection of 2-4 cell embryos on Day 2 of culture might help to improve the cloning efficiency following embryo transfer given the poor implantation rate of fragmented embryos (Ziebe et al., 1997; Ebner et al., 2001). Nevertheless, since we could not perform embryo transfer with these embryos, we do not know if 5 to 8 cell stage embryos retain some developmental ability and therefore, do not recommend their automatic discard. Our data may have implications in cases when there are large numbers of available embryos allowing an opportunity for selection. Our data is particularly important in the case of cloned porcine embryos because offspring rate from cloned embryos is very low (Yin et al., 2003; Park et al., 2005; Lee et al., 2007) and it requires large numbers of embryos to be transferred (typically 100 to 200 embryos per recipient) to assure that at least four blastocysts implant into the endometrium to sustain the pregnancy up to term (Lai et al., 2002; Park et al., 2005). Transferring greater numbers of 2 to 4 cell stage embryos to the recipients may help accomplish this objective.

In conclusion, our data suggest that a relationship exists between embryo morphology and their *in vitro* developmental competence. Morphological selection of embryos based on cell number on Day 2 of *in vitro* culture might be used as a valuable non-invasive criterion for selection of good quality embryos. Advances in pre-selection technique for normal and healthy porcine embryos could contribute to the improvement of cloned and transgenic animal production.

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