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RESEARCH ARTICLE

Day-3 Medium Changes can Affect Developmental Potential of Porcine Somatic Cell Nuclear Transfer and Parthenogenesis Embryos *In Vitro*

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ABSTRACT

The aim of the present study was to compare the developmental competence of porcine parthenotes and somatic cell nuclear transfer (SCNT) embryos after day-3 medium change with fresh embryo culture medium to that of embryos that did not have a medium change (monoculture system). The parthenogenetic and SCNT blastocyst formation rates were significantly ($P<0.05$) higher in the no-medium-change group (43.3±2.3, 18.5±1.1%, respectively) compared with the day-3 medium-change group (35.9±2.4, 7.9±0.9%, respectively). Total cell number in parthenotes and SCNT blastocysts was also significantly ($P<0.05$) higher in the no-media-change group (92.0±4.2, 66.9±7.7, respectively) compared with the media-change group (81.5±3.1, 46.6±4.9, respectively). No significant difference in cleavage rate was found in either group for parthenotes or SCNT embryos. This result suggests that day-3 medium changes have negative effects on porcine parthenotes and SCNT embryos *in vitro*.

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INTRODUCTION

Competence of any pre-implantation embryo through development up to blastocyst formation resulting from *in vitro* maturation, *in vitro* fertilization, and *in vitro* culture is affected by factors in the culture environment during the production of embryos in the laboratory. It has been suggested that a variety of molecules that are secreted by the embryo itself (e.g., ions, energy substrates, amino acids, vitamins, growth factors, cytokines, and hormones) play important roles in early embryonic development (Kuran *et al.*, 2002). The secreted growth factors are essential for further growth and development, and their absence can lead to implantation failure or, in rare cases, severe developmental abnormalities (Richter, 2008). Evidence also suggests that the addition of exogenous growth factors during *in vitro* embryo culture can influence developmental potential. Some genes [e.g., leukemia inhibitory factor (LIF) and interferon-tau (IFN-T)] are expressed by the early embryo of several species, and it has been shown that successful development and blastocyst quality is dependent upon the action of these growth factors (Stewart, 1994). Studies have shown that pre-implantation development-related gene knockout can result in embryonic lethality or severe post-implantation abnormalities. Injection of secreted factors can be a useful method to rescue the defects that cause embryonic lethality (Uehara *et al.*, 2000). Constant secretion and growth factor level in the medium are essential for successful *in vitro* embryo development. Alteration or changes in embryo-secreted growth factor levels could lead to developmental retardation. During *in vitro* embryo development, medium changes might alter developmental potential. Recently, sequential culture media have been used for *in vitro* porcine embryo development (Swain *et al.*, 2001). This technique is well documented in human *in vitro* embryo culture systems. The implantation and pregnancy rates of blastocysts cultured in a sequential media system are greater that those cultured under other conditions (Blake *et al.*, 2004). Conversely, conditions that support good growth of the zygote in culture do not sustain optimal blastocyst development and differentiation. Embryo-secreted growth factor concentration is critical and could help to further development into the blastocyst stage. However, no comparative studies have examined the developmental potential of *in vitro* embryos with and without (monoculture system) day-3 medium changes. The objective of the present study was to compare the developmental competence of *in vitro* embryo production through parthenogenetic and somatic

cell nuclear transfer (SCNT) technology using a day-3 porcine zygotic medium-3 (PZM-3)-change group and a no-medium-change group.

MATERIALS AND METHODS

Ovary collection, recovery and *in vitro* oocyte maturation

Ovaries of pre-pubertal gilts were collected from a commercial abattoir. The follicular contents were aspirated from 3- to 7-mm superficial antral follicles with a 10-ml disposable syringe and 20-gauge needle. Cumulus-oocyte complexes (COC) with at least three layers of compact cumulus cells and with homogenous cytoplasm were selected and a group of 50-60 COCs were cultured in tissue culture medium-199 (M-199) (Invtrogen, Grand Island, NY) supplemented with 0.6 mM cysteine, 0.91 mM pyruvate, 15 ng/ml epidermal growth factor, 75 µg/ml kanamycin, 1 µg/ml insulin and 10% porcine follicular fluid in each well of four-well multi dish (Nunc, Roskilde, Denmark). The COCs were then statically cultured at 39°C in a humidified atmosphere containing 5% CO_2 with 10 IU/ml eCG (Intervet International BV, Boxmeer, Holland) and 10 IU/ml hCG (Intervet International BV). After 20–22 h of maturation with hormones, the oocytes were washed two times in fresh maturation medium before being cultured in hormone-free medium for an additional 18 h for SCNT and 22 h for PA. The pFF was prepared according to Hyun *et al*. (2003) and stored at -20°C until use.

Donor cells preparation

Porcine ear skin fibroblasts from adult female pigs were seeded in to four-well plates and were grown in Dulbecco's modified Eagle medium (DMEM) with 1 mM sodium pyruvate, 1% (v/v) non-essential amino acids (Invitrogen, USA), and 10 µg/ml penicillin–streptomycin solution, which was supplemented with 10% (v/v) fetal bovine serum from a single batch until a complete monolayer of cells had formed. The donor cells were synchronized at the G0/G1 stage of the cell cycle by contact inhibition for 3–4 days. The cells of the same passage were used in each replicate for the various treatments. The individual cells were retrieved from the monolayer by trypsinization for ~1 min and subsequently used for SCNT.

SCNT and parthenogenesis

After 40 h of IVM, cumulus-cell-free oocytes were incubated for 2 min in manipulation medium (calcium free TLH-BSA) containing 5 µg/ml Hoechst 33343 and 7.5 µg/ml cytoclasin B (Sigma-Aldrich Co). Following incubation, the oocytes were transferred into a drop of manipulation medium containing 7.5 µg/ml cytoclasin B and were overlaid with warm mineral oil. The zona pellucida was partially dissected with a fine glass needle near the first polar body (PB). The first PB and adjacent cytoplasm (~10%), presumably containing the metaphase-II chromosomes, were extruded by squeezing the oocytes with the same needle. Enucleation was confirmed under an epifluorescence microscope (TE 300, Nikon, Tokyo, Japan). Using a injecting pipette, a 12–15-µm trypsinized fetal fibroblast with a smooth surface was transferred into the periviteline space through the same slit of an enucleated oocyte. The reconstructed couplets were equilibrated with 0.26 M mannitol containing 0.5 mM HEPES, 0.001 mM $CaCl_2$, and 0.05 mM $MgSO_4$ for 2–3 min and transferred to a 1 mm fusion chamber containing overlaid with same mannitol solution. Membrane fusion and activation were done according to Song *et al*. (2009). Activated oocytes were washed and cultured in PZM-3 medium supplemented with 3 mg/ml fatty-acid free BSA and placed in humidified incubator at 39°C under 5% CO_2.

For PA, the MII oocytes at 42 h of IVM were activated using a pulse sequence identical to that used to activate SCNT oocytes. The culture procedures of PA embryos were similar to SCNT embryos. Cleavage and blastocyst formation were evaluated at 48 and 168 h post activation, respectively, with the day of SCNT or PA designated Day 0.

Embryo evaluation and nuclear staining

Blastocysts considered viable were washed with 1% PVA in Dulbecco's phosphate buffered saline (DPBS) for 1 min and then fixed with 100% ethanol containing 10 µg/ml Hoechst for 5 min. Then, the blastocysts were mounted on glass slides in a drop of 100% glycerol and squashed gently with a coverslip. The nuclei were counted using fluorescence microscopy.

Embryo culture procedure

Following activation, the culture medium was changed on day 3 with fresh PZM-3 medium and cultured for further development. In the other group, the medium was unchanged up to day 7. At day 4 in both *in vitro* cultures (IVC), medium was supplemented with 10% FBS (final concentration) (Invitrogen, Carlsbad, CA).

The data were statistically analyzed by student *t*-test using GraphPad Version 5.0 with a probability level of $P<0.05$ being considered significant.

RESULTS

A total of 613 parthenogenetic activated embryos were randomly allocated into two groups of six replicates and were examined for developmental potential (Table 1). The parthenogenetic blastocyst formation rate and total cell number per blastocyst (Fig. 1: D) was significantly higher (43.3 ± 2.3%, 92.0 ± 4.2, respectively) in the no-medium-change group as compared with the medium change group (35.9 ± 2.4%, 81.5 ± 3.1, respectively). No significant difference in cleavage rate was observed between the two groups.

After SCNT, a total of 829 oocytes were injected in five replicates (Table 2). As shown in Table 2, the blastocyst formation rate and total cell number per blastocyst (Fig. 1: B) significantly increased (18.5±1.1%, 66.9±7.7, respectively) in the no-medium-change group compared with the medium change group (7.9±0.9%, 46.6±4.9, respectively). No significant difference in cleavage rate was found in either group for parthenotes or SCNT embryos.

DISCUSSION

A good embryological laboratory is a major factor in a successful assisted reproductive technology (ART) program. The choice of culture system used is a matter of individual preference and previous experience. Two

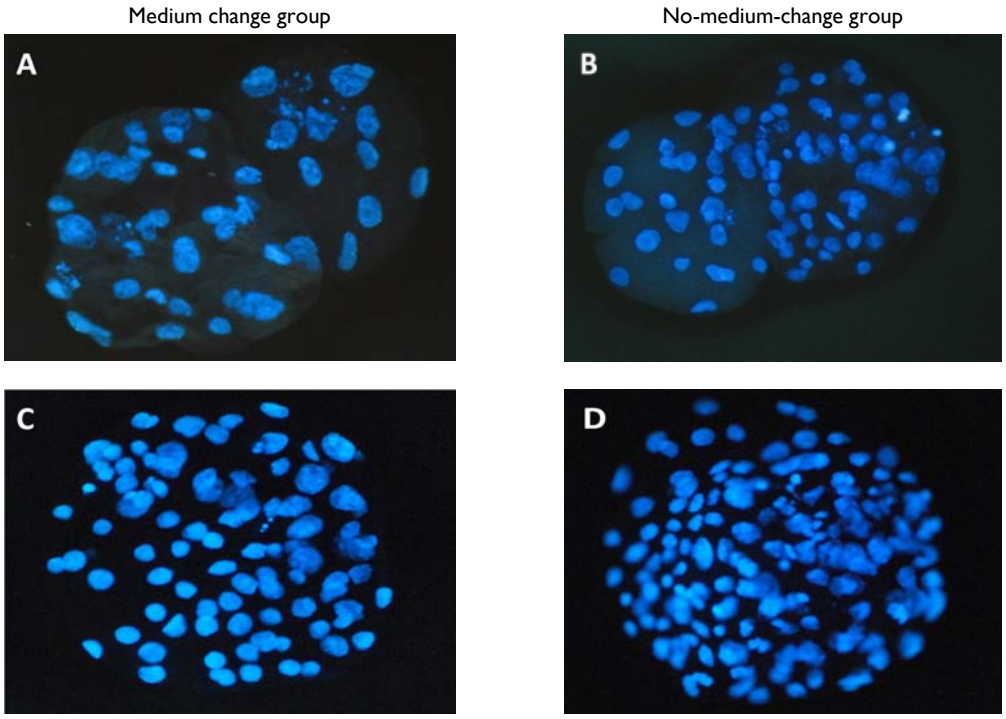

Fig. 1: Nuclei of blastocyst stained with Hoechst at 168 h after activation in medium change (A: SCNT and C: parthenotes) and no-medium-change group (B: SCNT and D: parthenotes) (x400).

Table 1: Effects of medium changes at day-3 on developmental potential of porcine *in vitro* parthenogenetic embryos

Group	Replications	Total oocytes cultured	Cleavage (%)	Blastocyst (%)*	Total cell number/BL (Lowest-Highest)
Medium change	6	314	257 (85.4±2.7)	92 (35.9±2.4)[a]	81.5±3.1[a] (36-134)
No medium change	6	299	258 (86.2±1.6)	111 (43.3±2.3)[b]	92.0±4.2[b] (41-167)

*Percentage of cleaved embryos. [a,b]Values with different superscripts in the same column are significantly different ($p<0.05$).

Table 2: Effects of medium changes at day 3 on developmental potential of porcine *in vitro* SCNT produced embryos.

Group	Repli-cations	Total oocytes injected	Fusion (%)	Cleavage (%)	Blastocyst (%)*	Total cell number/BL (Lowest-Highest)
Medium change	5	420	328 (77.9±1.7)	205 (62.6±3.2)	16 (7.9±0.9)[a]	46.6±4.9[a] (20-73)
No medium change	5	409	302 (73.1±1.8)	194 (63.8±3.5)	36 (18.5±1.1)[b]	66.9±7.7[b] (32-121)

* Percentage of cleaved embryos. [a,b] Values with different superscripts in the same column are significantly different ($P<0.05$).

methods are widely and successfully used under *in vitro* embryo culture, i.e., monoculture and sequential culture systems and a debate about the relative merits of *in vitro* culture systems continues. *In vitro* embryo culture medium was developed according to embryo metabolism. Sequential embryo culture mediums were used according to embryo requirements. Before embryo compaction, embryos have low metabolic activity and need a low level of glucose in a pyruvate-preferred nutrient medium (Flood and Wiebold, 1988). After compaction, embryos need a glucose-preferred nutrient medium, as well as an essential and non-essential amino acid-based medium (Lane and Gardner, 1997).

During *in vitro* embryo culture, the post-activation environment in which embryos are cultured is critical for normal development. This includes i) the first cleavage division, ii) the activation of the embryonic genome at the 8–16 cell stage, iii) compaction of the morula on day 5 and iv) blastocyst formation on day 6–7, which involves the trophoectoderm and the inner cell mass formation (Lonergan *et al.*, 2006). During this period, the embryo itself secretes growth factors that create a microenvironment that is essential for further growth and differentiation. Our study showed that day-3 medium changes with fresh medium without any supplementation altered the blastocyst formation rate and total cell number per blastocyst in parthenogenetic and SCNT embryos. This result is supported by another study (Swain *et al.*, 2001) in porcine *in vitro* fertilization (IVF) embryos, which found that the blastocyst formation rate was significantly lower in sequential culture medium than in monoculture medium. Therefore, it is clearly indicated that any modification of the culture environment that could affect any or all of these processes might have a major effect on embryo quality. During *in vitro* embryo culture, constant growth factor secretion and level is important. At that time, embryo-secreted growth factors act in a paracrine or autocrine fashion. Many attempts have been made to determine whether pre-implantation development, cleavage and differentiation of the blastocyst are influenced by endogenous or exogenous growth factors. The addition of growth factors (e.g., IGF-

2, insulin, platelet-derived growth factor-alpha, TGF-beta, EGF) to cultured pre-implantation embryos stimulates DNA and RNA synthesis and increases cell number (Richter, 2008). On the other hand, culture of mouse pre-implantation embryos at high density improves their developmental processes, perhaps due to the stimulatory effect of an increase in the local concentration of endogenously produced growth factors (Paria and Dey, 1990). Concentration of such autocrine growth factors is believed to be the primary reason for improved embryonic development with group culture and with minimization of medium volume (Jin *et al.*, 2001). This hypothesis is in agreement with our result that when the medium was changed at day 3 without adding any growth factor, the blastocyst formation rate and cell number per blastocyst in both cases declined compared with the no-medium-change condition.

During pre-implantation, developmental gene expression has a fundamental role in the coordination of homeostatic and metabolic mechanisms throughout life. In domestic species, some evidence suggests that culture environments post-fertilization or activation can perturb the gene expression pattern in pre-implantation embryos. During embryo culture, several genes known to be involved in important developmental process are expressed, including genes involved in apoptosis (Bax), gap junction formation (Cx43), and differentiation (LIF and LIF-Rβ) (Rizos *et al*., 2002). The post-activation culture environment can have a dramatic effect on the pattern of mRNA abundance of many developmentally important genes in the embryos. Supplementation of growth factors like IGF-I (Makarevich and Markkula, 2002) and EGF (Cui and Kim, 2003) in IVC culture medium has been shown to reduce apoptosis and increase cell number in porcine diploid parthenotes (Cui and Kim, 2003). This is similar to our results under the no-medium-change condition; total cell number increased in both cases. Even though, pre-implantation embryos express many growth factors, e.g. EGF, TGF-α, TGF-β, IGF-I, and II, VEGF, PDGF, PAF and fibronectin (Rappolee *et al.*, 1988; Richter, 2008), expression of a subset of growth factors in embryos suggests that they were involved in the growth and differentiation of early mammalian embryos (Rappolee *et al.*, 1988). In conclusion, our results suggest that using a monoculture system improved the developmental potential of porcine parthenogenetic and SCNT *in vitro* embryo production.

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