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FULL PAPER

Removal of cumulus cells before oocyte nuclear maturation enhances enucleation rates without affecting the developmental competence of porcine cloned embryos

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ABSTRACT

The present study compared the efficiency of somatic cell nuclear transfer (SCNT) using porcine oocytes that were denuded of their cumulus cells at different maturation time. In pre-denuded group, the cumulus cells from cumulus-oocyte complexes (COCs) were removed at 29 hr post *in vitro* maturation (hpm) and followed by further culture for 12 hr. In control group, as a commonly followed procedure, cumulus cells were removed from COCs at 41 hpm. The majority of porcine oocytes at 29 hpm were observed in metaphase of the first meiotic division (MI). At 41 hpm, no significant ($P > 0.05$) differences were observed in nuclear maturation and mitochondrial distribution of oocytes between pre-denuded and control groups. However, in pre-denuded group oocytes, metaphase II (MII) plate and spindle were located closely as 'adjacent' to the first polar body (PB1), resulting in an increased enucleation rates than in control group oocytes by blind enucleation method. Following SCNT and parthenogenesis (PA) using pre-denuded group and control group oocytes, no significant ($P > 0.05$) differences were observed with respect to the development, total cell number, incidence of apoptosis and the expression profile of developmentally important genes (Pou5f1, Dnmt1, Dnmt3a, Igf2r, Bax, Bcl2 and Glut1) at the blastocyst stage. In conclusion, the removal of cumulus cells at 29 hpm in porcine oocytes increased the enucleation rates through proper positioning of PB1 without compromising the quality of SCNT embryos during preimplantation development. Hence, this could be a valuable strategy to improve the SCNT efficiency in a porcine model.

Key words: Enucleation rates, gene expression, pig, removal of cumulus cells, somatic cell nuclear transfer

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Introduction

The success of pig cloning by somatic cell nuclear transfer (SCNT) promises extensive applications in agriculture and biomedical research in terms of the development of cloned animals for resource propagation, establishment of disease models and supplying organs for xenotransplantation[2,22,26]. However, the relative success of development to term by SCNT remains as low as 1–3%[2]. In this regard, several factors have been attributed to affect SCNT efficiency and amongst them, the oocytes activation protocol, *in vitro* embryo culture conditions, preimplantation developmental stage and transfer of embryos to surrogate recipients seem to be the critical steps involved in SCNT of pigs[22,31]. In addition, the cloning efficiency has been largely dependent on the process of enucleation, removal of metaphase II (MII) chromosome, meiotic spindle and the first polar body (PB1) from the *in vitro* matured oocytes[1,5]. Therefore, the success of SCNT can be further enhanced through the optimization of existing procedures used in pig cloning.

Currently, different methods of enucleation have been followed for the recipient oocyte[10,18], including blind enucleation[20], Hoechst-33342 induced enucleation[25], and chemical enucleation[6]. Each method of enucleation has its own set of merits and demerits. In ultra-violet (UV) excitable dye Hoechst-33342 staining method, the oocyte is subjected to enucleation after being exposed to stressful UV irradiation, which may have deleterious effect on cytoplasmic organelles and early embryo development[5,10]. With the chemical assistance, although the rate of enucleation was higher, reduced embryonic development was evident[7]. In blind enucleation method, location of MII chromosomes is indirectly determined by positioning of PB1[10]. Even though this method is relatively simple to follow, it has been reported that more than 10% residual oocytes DNA was noticed with in oocyte even after enucleation process[3,27]. The possibility of moving the PB1 by removing the cumulus cells from MII stage of oocytes offers a viable intervention to obtain high efficiency using conventional blind enucleation method[10]. Several studies have examined whether the presence or absence of cumulus cells during the maturation process affect the porcine oocyte quality and subsequent embryo development by *in vitro* fertilization[1,32,34]. Moreover, the use of bovine oocytes denuded of their cumulus cells prior to completion of MII stage and PB1 extrusion achieved rapid and effective enucleation of recipient cytoplast, and improved the developmental capacity of SCNT embryos[10]. However, there is very limited information on the removal of cumulus cells at different timings of porcine oocyte maturation and its implications for SCNT efficiency in pigs.

Therefore, the objective of the present study was to compare the efficiency of SCNT using porcine oocytes that have been denuded of their cumulus cells at different maturation time. In pre-denuded group, the cumulus cells from cumulus-oocyte complexes (COCs) were removed at 29 hr post *in vitro* maturation (hpm) and followed by further culture for 12 hr. At 29 hpm, we observed the majority of porcine oocytes (72.7%) in metaphase of the first meiotic division (MI). In previous studies, when the nuclear status of porcine oocytes matured for 33 hr was evaluated, 59%[12] and 76.1%[28] of the oocytes were at the MI stage. Hence, it is presumed that nuclear progression from MI to MII can be achieved after the removal of cumulus cells at this stage. In control group, as a commonly followed procedure, cumulus cells were removed from COCs at 41 hpm. Qualities of matured oocytes were evaluated by nuclear maturation rates and mitochondrial distribution. Relative distance and enucleation rates were also assessed to examine the protective effect against physical impact of cumulus cells removal. Furthermore, the developmental ability, incidence of apoptosis, total cell number, and the expression levels of developmentally relevant genes were analyzed

in the blastocysts obtained from SCNT and parthenogenesis (PA) using two groups of oocytes.

Materials and Methods

All experiments in this study were carried out following the guidelines of Animal Centre for Biomedical Experimentation at Gyeongsang National University.

Chemicals and media: Unless otherwise stated, all chemicals and media were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) and Gibco (Invitrogen Corporation, Grand Island, NY, USA), respectively. TCM199 supplemented with 0.1% (*w*/*v*) polyvinyl alcohol (PVA), 10 ng/ml (*w*/*v*) epidermal growth factor (EGF), 0.57 mM cysteine, 100 IU/ml penicillin G and 100 μg/ml streptomycin was used for *in vitro* maturation (IVM) of porcine oocytes. Nutrient mixture F-10 (Ham's F10) was used as washing medium for COCs. *In vitro* culture (IVC) of embryos was performed using porcine zygote medium-5 (PZM-5) as described previously[33]. Medium used for culture of fibroblasts was Dulbecco's modified eagle medium (DMEM) supplemented with 10% (*v*/*v*) fetal bovine serum (FBS), 0.1 mM 2-mercaptoethanol, 100 IU/ml penicillin and 100 μg/ml streptomycin. Dulbecco's phosphate buffered saline (DPBS) supplemented with 0.1 mg/ml $CaCl_2$, 0.1% (*w*/*v*) PVA, 100 IU/ml penicillin and 100 μg/ml streptomycin was used as washing medium. All media were adjusted pH to 7.4 and osmolality to 285 ± 5% mOsm/kg, and filtered using a 0.22 μm filter.

Preparation of donor cells: Porcine fetal fibroblasts (pFFs) were obtained from a miniature pig fetus at 29 days of gestation. After the removal of head, four limbs, tail and all internal organs using 29 G needle under a stereomicroscope, the remnant was washed in DPBS and enzymatically digested with 0.25% trypsin-ethylenediamine tetra acetic acid (trypsin-EDTA) solution for 5 min. Trypsinized cells were inactivated by adding 20% FBS, washed in DMEM supplemented with 10% FBS by centrifugation at 300 ×g for 10 min and cultured until confluent in DMEM containing 10% FBS at 38.5°C in a humidified atmosphere of 5% CO_2 in air. Cells at passage 3 were frozen in DMEM containing 20% FBS and 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen. For SCNT, frozen cells were thawed and cultured until they reached confluence.

In vitro maturation (IVM) of oocytes: Pre-pubertal porcine ovaries were obtained from a local slaughterhouse and carried to the laboratory within 2 hr. COCs from follicles (3–6 mm in diameter) were aspirated with 18 G needle attached to a disposable 10-ml syringe. After being washed twice with Ham's F10, only oocytes with uniform ooplasm and having three layers dense cumulus cells were selected. The chosen COCs were shifted into IVM medium supplemented with FSH (0.5 μg/ml) and LH (0.5 μg/ml). About 50 COCs were incubated in a 500 μl of maturation medium under a layer of mineral oil for 22 hr. Further, COCs were transferred to the fresh IVM medium without hormones and incubated for 19 hr. Cultures were performed in a 38.5°C humidified incubator with 5% CO_2 in air.

Removal of cumulus cells from oocytes: The effect of removal time of cumulus cells from COCs on the oocyte nuclear maturation were examined by randomly dividing COCs into two groups. In pre-denuded group, COCs were denuded by vortexing 2 min in 3% sodium citrate solution at 29 hpm. After the removal of cumulus cells from COCs, they were then allowed to mature further up to 41 hr. In control group, cumulus cells were removed only at 41 hpm. The cumulus-free oocytes with uniform ooplasm, intact cytoplasmic membrane and visible PB1 were selected for further experiments.

Oocyte nuclear position and mitochondrial distribution in matured oocytes: At 29 and 41

hpm, denuded oocytes were stained using 0.5 μg/ml Hoechst-33342 (prepared in DPBS) for 2 min at room temperature and assessed the oocyte nuclear conditions under a fluorescent microscope. At 29 hpm, oocytes were categorized as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI) and degenerated stages. Oocytes at 41 hpm were classified as immature, matured (MII) or undetermined. To observe mitochondrial distribution, the oocytes were incubated in a solution of DPBS supplemented with 10 μg/ml of rhodamine-123 (R-123) prepared in methanol for 15 min before overnight fixation in 4% paraformaldehyde. Using a mounting medium containing DPBS and glycerol (1:1), active mitochondria were observed under a confocal laser scanning microscope (FV-1000, Olympus, Japan).

Relative distance between PB1 and nucleus of MII oocytes: The relative distance between PB1 and nucleus of MII oocytes was determined using images taken after staining with Hoechst-33342 as mentioned previously[11]. Briefly, oocytes were determined as 'displaced' and 'adjacent' based on the distance between two nuclei which were less or more than 1/3 radius of the oocyte respectively (Fig. 1).

Somatic cell nuclear transfer (SCNT) and parthenogenesis (PA) embryo culture: SCNT was carried out by following previously described protocol with minor modifications[13]. Enucleation of recipient IVM oocytes of pre-denuded and control groups was performed by micromanipulation technique in the presence of HEPES-buffered TCM199 supplemented with 7.5 μg/ml cytochalasin B (CCB), 0.3% BSA and 12 mM sorbitol. Using 15 μm beveled micropipette, oocytes collected at 41 hpm were enucleated by aspirating PB1 and a small volume of adjacent cytoplasm presumably containing the MII chromosome. Successful enucleation was confirmed under an inverted microscope after staining with 0.5 μg/ml Hoechst-33342 for 2 min at room temperature. A single spherical donor cell was inserted into the perivitelline space and placed adjacent to the recipient cytoplasm of each of the enucleated oocyte. The reconstructed eggs were equilibrated in the fusion medium having 0.28 M mannitol solution containing 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$ and 0.01% BSA. Thus, the reconstructed eggs were fused and activated simultaneously with a twice DC pulse of 2.0 KV/cm for 30 μsec using an BTX Electro Square porator (ECM 830, BTX, Inc., San Diego, CA, USA). For parthenogenetic activation, medium and electric

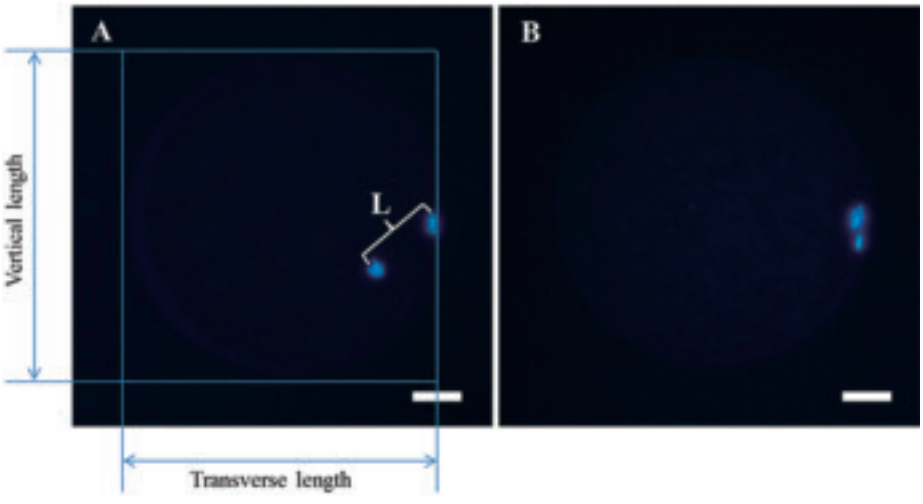

Fig. 1. Measurement of relative distance between PB1 and nucleus of the porcine MII oocytes. r. The radius of oocytes: vertical length/4 + transverse length/4, L. The distance between PB1 and MII. Matured oocytes were divided into two groups, such as 'displaced' (**A**: $L \geq 1/3r$) and 'adjacent' (**B**: $L < 1/3r$). Scale bar = 20 μm.

stimulus were used the same as SCNT. For embryo culture, sets of 30 fused eggs were placed in 30 μl droplet of PZM-5 medium for 2 days at 38.5°C in humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2, and further cultured in the same medium supplemented with 10% FBS for 5 days. The rates of cleavage and blastocysts formation were evaluated on Day 2 and 7 respectively (the day of SCNT performed was designated as Day 0). For the analysis of apoptosis and gene expression, blastocysts were collected on Day 7 and all the experiments were carried out in triplicates.

Apoptosis and total cell number in SCNT and PA blastocysts: DNA fragmentation of blastocysts was analyzed using TUNEL assay (in situ cell death detection kit, Roche, Germany) by a minor modification of the procedure described previously[9]. Briefly, blastocysts on Day 7 in each group were fixed overnight at 4°C in 4% (*w*/*v*) paraformaldehyde diluted in DPBS. Then the blastocysts were permeabilized with 0.5% Triton X-100 contained in 0.1% sodium citrate buffer for 1 hr. All the blastocysts were incubated in TUNEL reaction cocktail at 37°C for 1 hr in the dark. Further, blastocysts were incubated with RNase A (50 μg/ml) for 1 hr and counterstained with propidium iodide (PI, 50 μg/ml) for 1 hr and mounted slides with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA). The blastocysts were examined under a fluorescence microscope and those stained red (PI) were nucleus and those stained green and yellow (TUNEL reaction) were apoptotic body. Total cell numbers were also counted based on the nuclear staining by PI.

Gene expression in SCNT and PA blastocysts: Total RNA was extracted from pools of 20 blastocysts (triplicates) using RNeasy Micro kit (Qiagen, Hilden, Germany) in accordance with manufacturer's instructions. Reverse transcription-polymerase chain reaction (RT-PCR) was carried out in automated thermal cycler (Mastercycler® gradient Eppendorf AG, Hamburg, Germany) after cDNA synthesis using Sensiscript reverse transcription kit (Qiagen, Hilden, Germany). The sequence of the primers used, size of PCR product and the sequence references are presented in Table 1. Amplification was performed under the following conditions: 35 cycles with

Table 1. Primer sequences of gene transcripts used for RT-PCR

Gene	Primer sequence (5′–3′)	Product length (bp)	GeneBank Accession number
Pou5f1	**F**-AGGTGTTCAGCCAAACGACC **R**-TGATCGTTTGCCCTTCTGGC	335	TC205936
Dnmt1	**F**-TCGAACCAAAACGGCAGTAGT **R**-CGGTCAGTTTGTGTTGGAGAAG	215	NM_001032355
Dnmt3a	**F**-CTGAGAAGCCCAAGGTCAAG **R**-CAGCAGATGGTGCAGTAGGA	238	NM_001097437
Igf2r	**F**-CGCTCTCTGCCTCTAGCAGT **R**-CCTACACCCCAAGTCTGGAA	225	AF342812
Bax	**F**-ACACCTCATAGCCATGAAAC **R**-ATGGCTGACATCAAGATACC	232	AY550048
Bcl2	**F**-GAAACCCCTAGTGCCATCAA **R**-GGGACGTCAGGTCACTGAAT	196	NM_214285
Glut1	**F**-TTCTTTGAAGTGGGCCCTGGCC **R**-CAGTTGGAGAAGCCAGCGACGG	104	NM_214285
β-actin	**F**-CTCGATCATGAAGTGCGACG **R**-GTGATCTCCTTCTGCATCCTGTC	114	SSU07786

denaturation at 94°C for 30 sec, annealing at 52–62°C for 30 sec and extension at 72°C for 45 sec. The PCR products were separated by electrophoresis using 1% agarose gel with 0.1 mg/ml ethidium bromide. For the determination of gene expression levels, densitometric semi-quantitative RT-PCR analysis was performed using an image analysis program. Briefly, RT-PCR expression level for each mRNA of interest was normalized to β-actin mRNA levels and presented as a fold induction with mean ± standard deviation of mean (SD). Relative intensities of each specific band were assessed on the basis of corresponding β-actin intensity band by GelViewer Version 1.5 (CURVEX Corp., DAIHAN, South Korea).

Experimental design: In the experiment 1, denuded oocytes of pre-denuded and control groups were assessed for the status of nuclear development, mitochondrial distribution, and the relative distance between PB1 and nucleus of MII oocytes (Table 2, and Fig. 1 and 2). In the experiment 2, oocytes of pre-denuded and control groups were used for SCNT and PA and evaluated further for the rate of enucleation of oocytes, cleavage and development into blastocyst stage, and apoptosis and total cell number in blastocysts (Table 3). Finally in the experiment 3, SCNT and PA blastocysts produced from two groups of oocytes were analyzed for the expression of genes implicated in pluripotency (Pou5f1, also known as Oct4), DNA methylation (DNA methyltransferases, Dnmt1 and Dnmt3a), imprinting (insulin-like growth factor 2 receptor, Igf2r), apoptosis (Bax and Bcl2) and metabolism (glucose transporter 1, Glut1) (Fig. 3A and B). β-actin was used as an internal control.

Statistical analysis: Differences between groups were evaluated with Student *t*-test by SPSS 18.0 (SPSS Inc. Chicago, IL. USA). Data were expressed as mean ± SD, and probability value (P) of < 0.05 was considered to be significant.

Results

Nuclear maturation and mitochondrial distribution

At 29 hpm, the proportion of oocytes reaching MI stage (72.7 ± 5.2%) was higher than GV (7.2 ± 3.6%), GVBD (10.6 ± 2.2%) and degenerated stages (9.5 ± 4.6%) (mean ± SD, n = 113, triplicates). As presented in Table 2, at 41 hpm, there were no significant ($P > 0.05$) differences in nuclear developmental rates to MII stage between pre-denuded (70.2 ± 5.6%) and control group oocytes (68.5 ± 8.0%). Mitochondria were observed in the periphery of the ooplasm, and the fluorescence intensity of mitochondria did not differ between MII oocytes of pre-denuded and control group (Fig. 2).

Table 2. Nuclear maturation rates, the relative distance between PB1 and nucleus of MII oocytes, and enucleation rates in porcine oocytes

Oocytes Group	Experiment 1[a]		Experiment 2[b]
	% of MII	% of adjacent	% of Enucleated
Pre-denuded	70.2 ± 5.6 (302)	82.8 ± 4.0* (104)	87.7 ± 3.4* (1206)
Control	68.5 ± 8.0 (317)	74.7 ± 6.4 (109)	79.2 ± 6.9 (1281)

Pre-denuded: cumulus cells were removed from COCs at 29 hpm and further matured up to 41 hr; Control: oocytes were denuded at 41 hpm.
% values were mean ± SD of replicates.
Values in parentheses denote number of oocytes examined.
[a]7 replicates.
[b]12 replicates.
*Differ from the value of control group ($P < 0.05$).

Relative distance between PB1 and nucleus of MII oocytes

As presented in Table 2, the rate of 'adjacent' in pre-denuded group oocytes was significantly ($P < 0.05$) higher than that of control group oocytes. All the observed oocytes in this study exhibited 1/3 radius shorter than 20 μm.

Developmental efficiency of SCNT and PA embryos

There were significantly higher ($P < 0.05$) enucleation rates in SCNT pre-denuded group compared to SCNT control group (Table 2). However, no significant ($P > 0.05$) differences were found in fusion rates, cleavage rates and blastocyst rates of SCNT embryos between the two oocytes groups (Table 3). Similarly, PA embryos also showed no significant ($P > 0.05$) differences in cleavage rates and blastocyst rates between PA pre-denuded group and PA control group.

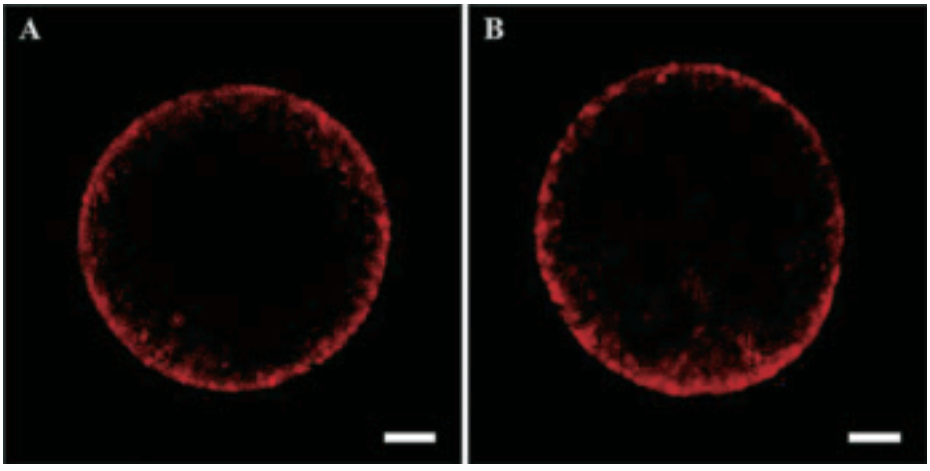

Fig. 2. Mitochondrial distribution of porcine MII oocytes. A: Oocytes were denuded at 29 hpm, and further cultured without cumulus cells up to 41 hpm (pre-denuded group); B: Oocytes were denuded at 41 hpm (control group). Scale bar = 20 μm.

Table 3. Fusion rate, developmental rate, total cell number and apoptosis in porcine SCNT and PA embryos

Oocytes group	% fused	% cleaved	% blastocysts	Cell number of blastocysts	% apoptosis
SCNT embryo					
Pre-denuded	63.5 ± 5.0 (12, 1054)	65.5 ± 7.2 (12, 671)	14.7 ± 3.6 (12, 671)	46.5 ± 3.5 (3, 32)	9.0 ± 0.8 (3, 32)
Control	62.4 ± 4.2 (12, 1023)	67.3 ± 5.5 (12, 636)	13.5 ± 2.7 (12, 636)	47.4 ± 3.7 (3, 25)	8.8 ± 1.0 (3, 25)
Parthenote embryo					
Pre-denuded	–	85.0 ± 3.4 (7, 1511)	25.4 ± 3.1 (7, 1511)	60.5 ± 2.8 (3, 36)	4.2 ± 0.5 (3, 36)
Control	–	85.7 ± 4.9 (7, 1608)	24.4 ± 3.7 (7, 1608)	61.2 ± 3.5 (3, 33)	4.3 ± 0.4 (3, 33)

Pre-denuded: cumulus cells were removed from COCs at 29 hpm and further matured up to 41 hr; Control: oocytes were denuded at 41 hpm.
% values were mean ± SD of replicates.
Values in parentheses denote number of replicates and total number of oocytes or embryos examined.

Apoptosis and total cell number of SCNT and PA blastocysts

As presented in Table 3, the mean total cell numbers of day-7 blastocysts in SCNT pre-denuded group and control group, and in PA pre-denuded group and control group did not differ significantly ($P > 0.05$). Similarly, there were no significant ($P > 0.05$) differences in apoptotic incidence of day-7 blastocysts in SCNT pre-denuded group and control group, and in PA pre-denuded group and control group.

Expression of developmentally important genes in SCNT and PA blastocysts

Representative RT-PCR gel photographs and the mean values obtained for the calculated relative expression of the selected gene transcripts are shown in Fig. 3A and B, respectively. The mRNA expressions of all selected genes implicated in various functions of early embryonic development were detectable in SCNT pre-denuded group and control group and PA pre-denuded group and control group blastocysts. The relative abundances of selected genes (Pou5f1, Dnmt1, Dnmt3a, Igf2r, Bax, Bcl2 and Glut1) were unaffected at the blastocyst stage of SCNT pre-denuded group and control group, regardless of whether the recipient oocytes were matured with or without cumulus cells. Further, the blastocysts of PA pre-denuded group also

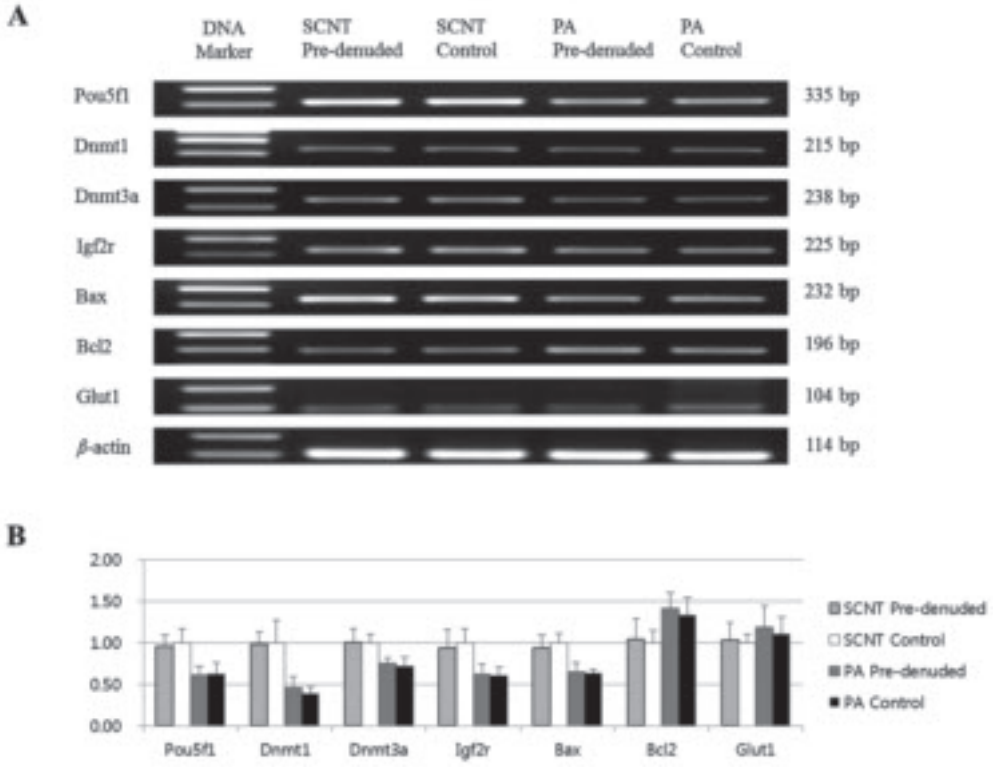

Fig. 3. Expression of developmentally important genes in SCNT and PA blastocysts by reverse transcription-polymerase chain reaction (RT-PCR). A: Selected genes, such as Pou5f1, Dnmt1, Dnmt3a, Igf2r, Bax, Bcl2 and Glut1 were detected in SCNT and PA blastocysts produced from pre-denuded group and control group oocytes. β-actin transcript was used as an internal control. B: The relative expression of selected genes in SCNT and PA blastocysts is shown by semi-quantitative RT-PCR analysis. RT-PCR expression level for each gene was normalized to β-actin mRNA levels and presented as a fold induction with mean ± SD. SCNT pre-denuded group: somatic cell nuclear transfer (SCNT) was carried out with pre-denuded group oocytes; SCNT control group: SCNT was carried out with control group oocytes; PA pre-denuded group: parthenogenesis (PA) was carried out with pre-denuded group oocytes; PA control group: PA was carried out with control group oocytes; pre-denuded group: cumulus cells were removed from COCs at 29 hpm and further matured up to 41 hr; control group: oocytes were denuded at 41 hpm.

displayed similar levels of gene expression compared with their PA control group counterparts.

Discussion

For the successful production of SCNT embryos, MII oocytes are generally used as recipient oocytes, and optimal maturation of these oocytes is demonstrated as one of the important factors influencing their developmental competency. Previous studies have suggested a positive correlation between MII oocytes nuclear and cytoplasmic maturation upon co-culturing with cumulus cells[29,34]. Keeping the synergistic role of cumulus cells, in this study, cumulus cells were removed from oocytes initially at 29 hpm and they were further allowed for 12 hr of IVM without cumulus cells in pre-denuded group. In control group, IVM of oocytes was carried out for 41 hr in the presence of cumulus cells. Our results indicated that most of the denuded oocytes had their nuclear status as MI at 29 hpm and these observations are in accordance with previous reports[12,28]. Further, when oocytes were allowed for 12 hr of IVM without cumulus cells, no significant differences were found in the rate of nuclear development to MII stage and mitochondrial distribution of oocytes when compared to control group. However, the relative distance between PB1 and nucleus of MII oocytes as termed as 'adjacent' was higher in pre-denuded group oocytes than that of control group oocytes. Finally, when SCNT and PA embryos were produced by using two groups of oocytes, except for enucleation rates, the removal of cumulus cells at 29 hpm did not influence any differences in the rates of cleavage and blastocyst development, total cell number, apoptosis, and the expression of selected genes at the blastocyst stage.

Mitochondria in oocytes play a critical role in supplying ATP (adenosine triphosphate) during mammalian preimplantation embryo development, and their distribution in cytoplasm is a crucial factor for further successful development of embryos[30]. It has been shown that, mitochondria get congregated and impaired in their function after the removal of cumulus cells from oocytes, but it can revert to normal condition by co-culturing with cumulus cells[8]. Recently, mitochondrial distribution in the cortical area of the ooplasm has been reported in porcine COCs and denuded oocytes[34]. Supporting these observations, in our study, the mitochondria were located at the periphery of the ooplasm in both pre-denuded and control group oocytes. However, there was no difference in the distribution of mitochondria between the oocytes of pre-denuded group and control group. Therefore, it indicates that the removal of cumulus cells at 29 hpm and further allowing 12 hr of IVM without cumulus cells has no detrimental effects on nuclear and cytoplasmic maturation of MII oocytes.

Out of several enucleation procedures, blind enucleation is the most commonly practiced method followed in SCNT technique. However, in the blind enucleation procedure, aspiration of PB1 and adjacent cytoplasm does not guarantee the adequate removal of chromatin, because the PB1 gets frequently displaced from the original location of expulsion[10,21,24]. Although the denudation process is one of the main reasons for the displacement of PB1, the exact cause is still unclear. The removal of cumulus cells is required prior to oocyte manipulation and it is commonly being carried out either by vortexing or pipetting. These physical impacts shift the location of the PB1 in the perivitelline space of oocyte[1]. In this view, removal of cumulus cells prior to the extrusion of PB1 in pre-denuded oocytes certainly prevented its relocation as observed previously[10]. Further, a high rate of enucleation was achieved by blind aspiration using pre-denuded oocytes (89.5%) than those from normal oocytes (41.9%)[10]. Moreover, it has been shown that the MII chromosome plate was located underneath the PB1 in most of the oocytes denuded of cumulus cells prior to the PB1 extrusion[10].

Therefore, when the PB1 was subsequently extruded in the perivitelline space, it was observed to be located "adjacent", and thus, resulting in enhanced enucleation rates during SCNT when compared to control group oocytes. Apart from efficient enucleation process, the usage of oocytes from pre-denuded group could possibly reduce the complexities of aneuploidy formation and parthenogenetic activation[5].

In this study, the efficiency of oocytes from pre-denuded and control groups to undergo cleavage and embryonic development to reach the blastocyst stage was statistically comparable, when they were used for both SCNT and PA. These results indicate that the removal of cumulus cells at 29 hr of porcine IVM appeared to have no impact on the developmental rates of embryos in the present IVM condition. Additionally, it is well known that the embryo quality and postimplantation developmental potential are likely to be affected not only by adequate cell numbers[19], but also apoptotic incidence in the preimplantation embryos[9]. Apoptosis plays a crucial role in the cellular response to suboptimal developmental conditions and stress[11]. For these reasons, the incidence of apoptosis has been regarded as an important parameter in the evaluation of embryo quality[9]. The results of this study clearly showed that, the total cell numbers and apoptosis incidence in SCNT and PA embryos were not affected by the removal of cumulus cells at 29 hpm.

Preimplantation development of porcine embryos has been shown to be influenced by the events occurring at maturation process and hence, the gene expression in SCNT and PA embryos produced from two groups of oocytes was examined to assess the embryo quality and viability. Our results showed that the expression levels of all selected genes were similar in SCNT and PA blastocysts produced using oocytes from pre-denuded and control groups.

Pou5f1 (Oct4) is a central regulator of pluripotency across mammalian species and is shown to be a critical factor controlling murine, bovine and porcine preimplantation embryonic development[14]. In this study, the expression of Pou5f1 in both groups of SCNT blastocysts demonstrated its possible participation in the regulatory circuit of transcription factors required for porcine embryo development. Therefore, the removal of cumulus cells at 29 hpm did not affect the expression of Pou5f1 transcript in SCNT and PA blastocysts.

Abnormal expression of Dnmt1 and Dnmt3a was reported to contribute to the compromised development of *in vitro* produced and cloned porcine embryos[11,15]. In this study, SCNT and PA blastocysts from pre-denuded group and control group oocytes seemed to have identical levels of Dnmt1 and Dnmt3a transcripts, suggesting that their similar developmental rates could be predicted in terms of comparable nuclear reprogramming.

Igf2r is a paternally imprinted gene implicated in growth of the fetus, and is essential for normal development[16]. Previous studies have demonstrated the utility of Igf2r gene expression profile in assessing the quality of porcine embryos[15,20]. The present results of Igf2r expression reveal that SCNT embryos from two groups of oocytes possess similar qualities that are important for fetal growth regulation during postimplantation development.

In porcine parthenotes, higher blastocyst rates and cell numbers were corresponded with reduced apoptotic cell death and Bax expression, and increased Bcl2 expression[4]. Indicating the superior quality, lower expression of Bax and higher expression of Bcl2 were evident in blastocysts derived from *in vivo* when compared to parthenote, *in vitro* fertilized and SCNT blastocysts[15,20]. In agreement with these findings, in this study, the expression levels of Bax and Bcl2 in SCNT and PA blastocysts supported the results observed with regard to the developmental rates, total cell number and apoptosis.

The analysis of Glut1 expression as a metabolic protein has been considered with high importance in growth and development of an

embryo[17]. Glut1 mediates the cellular glucose incorporation into embryonic cells and its expression has been detected during the preimplantation embryo development[17,23]. In this study, the removal of cumulus cells at 29 hpm was shown to have no effect on Glut1 expression in both SCNT and PA blastocysts.

In conclusion, the present study demonstrated that the removal of cumulus cells at 29 hpm did not displace the position of PB1 and further, it significantly improved the enucleation rates during SCNT. However, no marked differences were observed in the two groups of oocytes with respect to the nuclear maturation rate and mitochondrial distribution. In addition, following SCNT and PA, no significant differences were observed with respect to the developmental potential, total cell number, incidence of apoptosis, and the expression of developmentally relevant genes at the blastocyst stage. Hence, this could be a valuable strategy to improve the efficiency of SCNT technique in a porcine model.

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Disclosure of potential conflicts of interest

The authors indicate no potential conflicts of interest.

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