Effect of Cultured Cumulus Cells as Donor Cells on Reconstructed Porcine Oocytes

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ABSTRACT

The effects of cultured cumulus cells as donor cells on the genetically reconstructed porcine oocytes, matured *in vitro,* after nuclear transfer were studied. The cultured cumulus cells (F_4) and the freshly acquaired cumulus cells (F_0) were injected into the perivitelline space of the enucleated oocytes to obtain the genetically reconstructed oocytes.   The oocytes were fused and activated simultaneously. These oocytes were observed for the embryo development for 144 h. It was found that the activation rate and the one-pronuclear formation (1N) of the F_4 group were significantly higher than the F_0 group ($p<0.05$). Also, the embryo development rate of the F_4 group was significantly higher than the F_0 group ($p<0.05$). In addition, the

cultured cumulus cells enhanced the genetically reconstructed oocytes to advance their development to such stage as morula. These effects were attributed a better synchronization of the donor cells with the recipient cells of the F_4 group.

Keywords: Porcine oocyte, *In vitro* maturation, Cumulus cells, Nuclear transfer, Embryo development.

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以不同來源之卵丘細胞為供核細胞對重組豬卵母細胞之影響

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摘要

　　本研究乃探討以不同期別之卵丘細胞為供核細胞對體外成熟豬卵母細胞核移置重組卵之影響。將新鮮卵丘細胞 (F_0) 與繼養第 4 代卵丘細胞 (F_4) 分別注入於去核體外成熟豬卵母細胞之卵黃囊膜下間隙以獲得核移置重組卵。重組卵同時進行融合與激活,並培養 144 小時觀察胚發育情形。結果發現,F_4 組之激活率與 1N 原核形成率顯著高於 F_0 組 (p<0.05),且 F_4 組之胚發育率也顯著高於 F_0 組 (p<0.05)。此外,也發現卵丘細胞經繼代培養後有利於重組卵發育到桑椹胚階段,可能原因為 F_4 組之卵丘細胞與受核細胞有較佳之細胞周期同期化所致。

關鍵詞 ： 豬卵母細胞、體外成熟、卵丘細胞、核移置、胚發育。

I. Introduction

　　Since Dolly was successfully cloned by the use of adult sheep's mammary gland cells to demonstrate nuclear transfer (Wilmut et al., 1997), studies on cloning of animals were inspired all over the world. Various animals including mouse (Schuetz et al., 1996; Wakayama et al., 1998), cattle (Cibelli et al., 1998) and goat (Baguisi et al., 1999) were cloned through nuclear transfer. Various somatic cells including sheep mammary gland epithelial cells (Wilmut et al., 1997), male mice sertoli cells and

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brain neuronal cells (Wakayama et al., 1998), male mice tail somatic cells (Wakayama and Yanagimachi, 1999), granulus cells of cattle follicular (Wells et al., 1999), cattle ear fibroblast cells (Kubota et al., 2000), porcine cumulus cells (Uhm et al., 2000), cattle oviduct epithelial cells (Saikhun et al., 2002), adult cat fibroblast cells (Kitiyanant et al., 2003), porcine featal fibroblast cells (Kwon et al., 2007) and porcine ear fibroblast cells (Lin et al., 2008) were used as donor cells in the process of nuclear transfer. Among these somatic cells, cumulus cells and oocytes were demonstrated to have a strong, intimate relationship. The oocyte maturation is dependent on the existing of cumulus cells (Fukui, 1990); thus, cumulus cells play an important role in maturation procedure for female mammalian oocytes (Mori et al., 2000).

Cumulus cells are located around periphery of a primary oocyte, and are in contact with the oocyte at gap junction (Coskun and Lin, 1994). The necessary nutrition of an oocyte such as ions, nucleotides and amino acids of low molecular weight matter (<1000 Mr) can be transported into the oocyte through the gap junction (Kalous et al., 1993). The gap junction is known to play an important role in cell proliferation, differentiation and balance regulation of oocyte. When cumulus cells receive the stimuli of gonadotropin (LH), they secrete hyaluronic acid, which causes mucification and expansion phenomenon to occur (Nakayama et al., 1996). The moderate expansion of cumulus cells is necessary for normal ovulation and subsequent fertilization (Vanderhyden and Armstrong, 1989). In addition, cumulus cells are beneficial to the formation of a male pronucleus of sperm in an oocyte after fertilization (Ka et al., 1997). Thus, the advantages of the use of cumulus cells as donor cells in cloning animals are obvious. Since porcine oocytes are very sensitive to temperature, the developmental competence of nuclear transfer is low, and the production probability of cloned porcine is also low. In other words, it is more

difficult to produce cloned pigs than other livestocks. The use of cumulus cell may enhance the chance of clone for pigs. However, little was known about the detailed relationship between the cumulus cells and the cloning of pigs through nuclear transfer (Terlouw et al., 1992). In this study, the cumulus cells were used as the source of donor cells to investigate their effects on the genetically reconstructed porcine oocytes, matured *in vitro,* after nuclear transfer. Also investigated were their effects on the activation and embryo developmental competence of the genetically reconstructed porcine oocytes.

II. Materials and Methods

2-1 Preparation of Supplemented Maturation Culture Medium

The NCSU-23 maturation culture medium was prepared according to Wang et al. (1997). In this study, the NCSU-23 medium was supplemented with 0.5 μg/ml estradiol (E$_2$), 2.5 μg/ml follicle-stimulating hormone (FSH), 5 IU/ml luteinizing hormone (LH), 20 ng/ml luteotropic hormone (LTH) and 20% porcine follicular fluid (pFF). The medium was filtered with 0.2 μm filtration membrane (Minisart®, Sartorius), and stored in a refrigerator at 4℃

2-2 Collection of Cumulus-Oocyte Complexes (COCs) and *In Vitro* Maturation Culture

Porcine ovaries were collected from a local slaughterhouse and transported to the laboratory within 30 min in Dulbecco's phosphate saline (PBS) at 35–37℃. The ovaries were placed in a dish (6cm diameter) containing PBS. COCs were obtained by

removing the antral follicles (3–5 mm in diameter) with a dissection blade at room temperature. The COCs surrounded with more than three layers of intact cumulus cells were selected, washed three times with PBS, and then further washed one time with the supplemented NCSU-23. The COCs (about 10 COCs in each 50 μl microdrop) were cultured in the supplemented NCSU-23, which was covered with 2 ml mineral oil, and incubated in a 95-100% humidified atmosphere of 5% CO_2 in air for 48 h.

2-3 Preparation of Donor Cells

2-3-1 Fresh (F_0) Cumulus Cells

After maturation, cumulus cells were removed by vortexing the COCs in PBS containing 0.1% (w/v) hyaluronidase (Sigma-Aldrich). The separated cumulus cells were washed twice in PBS and twice in 2 ml DMEM (Dulbecco's Modified Eagle's Medium; GIBCO DMEM-11965) supplemented with 10 % fetal calf serum (FCS) and centrifuged at 600 x g for 5 min. Some of these cumulus cells were used as donor nuclei and thus treated as fresh cumulus cells (F_0).

2-3-2 Preparation of Passage 4 (F_4) Cumulus Cells

The separated cumulus cells were cultured in DMEM supplemented with 10 % FCS, in a 95–100% humidified atmosphere of 5% CO_2 in air at 39℃. The cell confluence was achieved within 3–5 days, and then were trypsinized and cultured again in DMEM supplemented with 10 % FCS. When cumulus cells were cultured up to 4 passages, they were used as donor cells for nuclear transfer.

2-4 Preparation of Recipient Cytoplasm

The separated matured oocytes were placed in micromanipulation PBS containing 0.1% (w/v) hyaluronidase (Sigma-Aldrich) medium. The oocytes were enucleated by aspirating the polar body and the metaphase-II plate in a small volume of surrounding cytoplasm. Enucleation was confirmed by staining the aspirated polar body and the metaphase-II plate with 1 μg/ml bisbenzzimide (Hoechst 33342, Sigma-Aldrich) for 15-20 min at 37℃.

2-5 Nuclear Transfer, Fusion and Activation

An operation procedure of nuclear transfer described by Zakhartchenko et al. (1999a) was used. A single cumulus cell was sucked into a micro injection pipette. Then, the cell was deposited into the perivitelline space of an enucleated oocyte through the split during enucleation. The genetically reconstructed oocytes were placed to an electric fusion medium, containing 0.3 M mannitol (Sigma), 0.1 mM CaCl$_2$ (Sigma); and 0.1 mM MgSO$_4$ (Sigma), left standing for 5 min, and then transferred into an electric fusion chamber with two electrodes separated by 0.5 mm (No. 450; BTX Inc, fusion chamber). Fusion and activation were carried out simultaneously at 5 V AC for 10 sec and DC pulses of 2.0 kV/cm for 30 μsec (Hyun et al., 2003).

2-6 *In Vitro* Culture of Reconstructed Oocytes

The genetically reconstructed oocytes after electrical fusion were washed three

times with NCSU23 embryo culture medium (Machaty et al., 1998). Then, 10 oocytes

in 50 μl of NCSU23, covered with 2 ml mineral oil, were cultured in a 95－100%

humidified atmosphere of 5% CO_2 in air at 39℃ for 16 h for the activation or 144 h

for the embryo development. After staining with Hoechst 33342 medium, the

pronuclear formation and embryo development phenomena of reconstructed oocytes

were observed and recorded.

2-7 Characterizations

2-7-1 Fixing and Staining

The reconstructed oocytes or embryos were stained with 10 μ g/ml

bisbenzzimide (Hoechst 33342, Sigma-Aldrich) medium for 20 min, and the cell

numbers were counted at a wavelength of 343 nm of ultraviolet. The reconstructed

oocytes or embryos were mounted on glass slides, and covered with a cover glass

supported by droplets of a paraffin-petroleum jelly mixture (1:1, v/v). Then, the

mounted oocytes or embryos were fixed with a mixture of anhydrous alcohol/glacial

acetic acid (1:3, v/v) for 48h and stained with 1% lacmoid according to the method

described by Abeydeera et al. (1998). The pronuclear formation and embryo

developmental competence of reconstructed oocytes or embryos were examined under

a phase-contrast microscope.

2-7-2 Evaluation of Pronuclear Formation of Reconstructed Oocytes

The pronuclear formation of reconstructed oocytes was examined according to

the method described by Zakhartchenko et al. (1999b). A reconstructed oocyte was

judged to be activated when pronuclear formation was found in it.

2-7-3 Evaluation of Reconstructed Embryos

The embryo development stage of reconstructed embryos was determined according to the method described by Lee et al. (2003). The reconstructed embryos were placed on an inverted phase-contrast microscope to discern the number of blastomeres, and were then fixed and stained to confirm the nucleus number of blastomeres.

2-8 Statistical Analysis

There were at least three replicates for each treatment. Data of pronuclear formation and embryo development of reconstructed oocytes were analyzed by ANOVA in SAS (1999) software. Differences at $p<0.05$ were considered to be statistically significant.

III. Results

3-1 Effect of Cultured Cumulus Cells as Donor Cells on the Activation of the Reconstructed *In Vitro* Matured Porcine Oocytes

After fusion, the reconstructed oocytes were cultured in NCSU-23 embryo medium for 16 h to check the activation and pronuclear formation. If no pronuclear formation was found after staining, the reconstructed oocytes were not activated. However, if only one pronucleus was formed, typically as shown in Figure 1(a), the

reconstructed oocytes were noted as activated successfully. Sometimes, part of the nuclear was not completely squeezed out, the residual chromosomes might be activated to form another pronucleus. Figure 1(b) shows typical two-pronuclear (2N) morphology. The two-pronuclear (2N) reconstructed oocytes, though activated, would be unfavorable for further embryo development.

Table 1 summarizes the activation of the reconstructed oocytes with cumulus cells as donor cells. The activation rate of the F_4 group (53.1%) was significantly higher than the F_0 group (41.0%), ($p<0.05$). The one pronuclear formation rate of the F_4 group (34.1%) was also significantly higher than that of the F_0 group ($p<0.05$).

3-2 Effect of Cultured Cumulus Cells as Donor Cells on the Developmental Competence of the Reconstructed Embryo

After fusion, the reconstructed oocytes were cultured in NCSU-23 for 144 h the embryo development. Figure 2 shows the different developed stages of reconstructed embryos.

.Table 2 summarizes the development phenomena of the reconstructed embryos. As shown in Table 2, various degrees of the development of the reconstructed embryos were recorded; some failed to develop and others developed to different stages, such as 2 cells, 4 cells, 8 cells or morula. The development rate of the F_4 group (45.4%) was significantly higher than that of the F_0 group (39.7%), ($p<0.05$). The embryo development rate of the F_4 group (13.5%) was also significantly higher than that of the F_0 group (5.5%), ($p<0.05$) at the stage of 8 cells or more.

In addition, no morula could be found in the F_0 group, indicating that its advanced development might be inhibited. Contrarily, the development of the F_4 group could proceed through the stages until reaching the morula stage. Thus, the

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cultured cumulus cells as donor cell could enhance the advanced development of the reconstructed embryos.

IV. Discussion

The effects of various cultured somatic cells as the donor cells on the development of reconstructed oocytes were well documented (Zakhartchenko et al. 1999b; Kitiyanant et al. 2003). Although some studies showed little effect (Hill et al. 2000), the cultured cells as donor cells did enhance the development of the reconstructed oocytes after nuclear transfer as reported by Zakhartchenko et al. (1999b), Saikhun et al. (2002), and Kitiyanant et al. (2003). This was mainly described as the effect of the cell cycle (Kitiyanant *et al.* 2003).

Hayes et al. (2005) reported that the cell cycle stages include G_0 (growth arrest phase), G_1 (synthesis prophase of DNA), S (synthesis phase of DNA), G_2 (preparation for mitosis) and M (mitosis phase). The donor cells and the recipient cells were situated at their respective cell cycle stages, if the cell cycle stage of the donor cells and that of the recipient cells were not synchronized, the activation and pronuclear formation of the reconstructed oocytes may be inhibited. However, if the cell cycle stages of the donor cells and the recipient cells were synchronized at G_0 or G_1 phase, the activation, pronuclear (1N) formation and subsequent embryo development of the reconstructed oocytes might be enhanced.

The activation rate and the pronuclear (1N) formation rate of the F_4 group were significantly higher than that of the F_0 group in this experiment. It is similar to the case described by Zakhartchenko et al. (1999b). It was possible that the cell cycle of most of the F_0 cumulus cells was at S phase, and that of the F_4 cumulus cells was at G_0 or G_1 phase. After nuclear transfer, the cell cycle of the F_4 cumulus cells was more

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synchronized with that of the recipient cells than the F_0 cumulus cells. Therefore, it was easier for F_4 nuclei to induce reprogramming by oocytes, and returned to the original position for developing (Campbell, 1999). Thus, the activation and the pronuclear (1N) formation of the F_4 group were significantly increased.

In this study, the reconstructed embryo development rate of the F_4 group was significantly higher than that of the F_0 group (Table 2). Furthermore, the cultured cumulus cells favored the embryo development. This may be due to that the cell cycle of the F_4 cumulus cells was more synchronized with that of the recipient oocyte cells than the F_0 cumulus cells. Additional studies will be made to further improve the development of the reconstructed porcine embryos cultured *in vitro.*

V. Conclusions

Using the cultured cumulus cells as donor cells, this study has successfully enhanced the rates of activation, pronuclear formation and embryo development of the reconstructed porcine oocytes after nuclear transfer. This success was possibly attributed to a better synchronization in cell cycles between the donor cells and the recipient cells.

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Table 1 - Effect of cultured cumulus cells as donor cells on the activation of reconstructed *in vitro* matured porcine oocytes.

Cultured passages	No. of oocytes examined (Replicates)	Total activation (%) (Mean±S.E.M.)	1 Pronucleus (%) (Mean±S.E.M.)	2 Pronuclei (%) (Mean±S.E.M.)
Fo	78 (3)	41.0 ± 1.2^{b}	24.4 ± 4.6^{b}	16.6 ± 5.8^{a}
F_4	111 (3)	53.1 ± 5.5^{a}	34.1 ± 2.8^{a}	19.0 ± 7.2^{a}

*Different superscripts (a, b) in the same column indicate significant differences ($p<0.05$).

Table 2 - Effect of cultured cumulus cells as donor cells on the developmental competence of reconstructed embryos.

Cultured passages	No. of oocytes examined (Replicates)	No. of embryos development (%) (Mean±S.E.M.)	2 cell (%) (Mean± S.E.M.)	4 cell (%) (Mean± S.E.M.)	8 cell (%) (Mean± S.E.M.)	Morula (%) (Mean± S.E.M.)
F_0	100 (3)	40 (39.7±2.0)[b]	13.2±10.0[a]	21.0±12.6[a]	5.5±4.6[a]	0.0±0.0[a]
F_4	82 (3)	37 (45.4±1.8)[a]	13.4±7.4[a]	18.6±8.0[a]	10.3±7.9[a]	3.2±3.1[a]

*Different superscripts (a, b) in the same column indicate significant differences ($p < 0.05$).
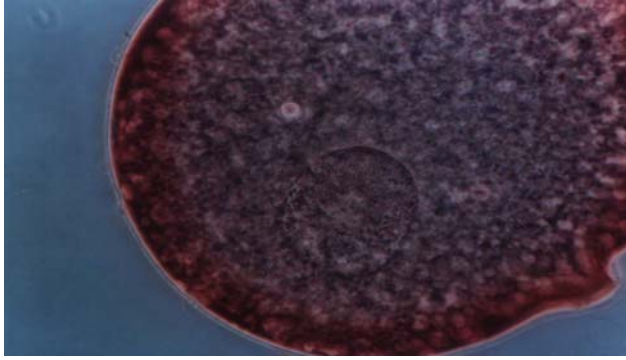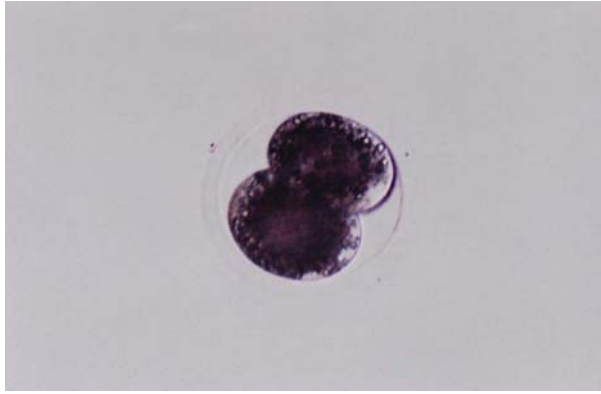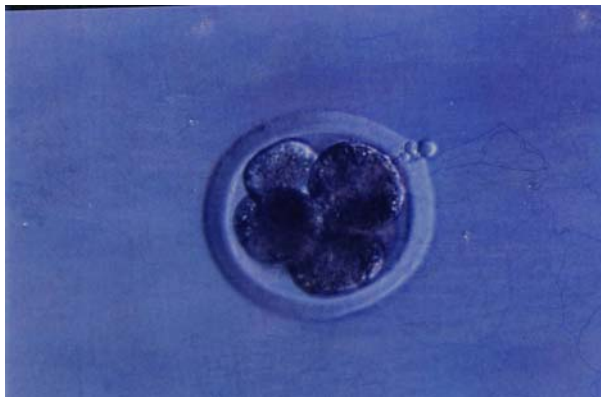
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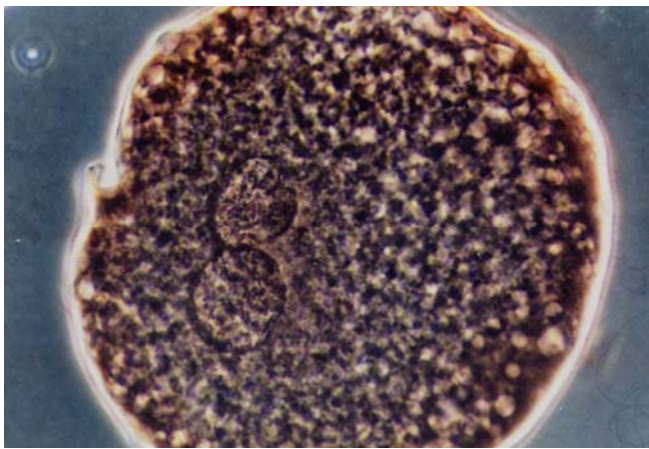
(b)

Figure 1 - Morphology of typical reconstructed oocytes with 1 pronucleus (1N) (a)

and 2 pronuclei (b) after fusion. (600X)

(a)

(b)

(c)

Figure 2 - Morphology of different develomental stages of reconstructed embryos: (a) 2 cells, (b) 4 cells, and (c) morula. (600X)