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Bovine Nuclear Transfer Using Primary Cultured Cumulus Cells of Determined Cell Cycle-Phase

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Abstract: Normal development of nuclear transfer embryos is thought to be dependent on transferal of nuclei in GO or G1 phases of the cell cycle. Therefore, the objective of this study was to investigate the cell cycle phase of different sizes of bovine cumulus cells cultured under serum starvation conditions and the consequent development after nuclear transfer. Cumulus cells after oocyte maturation were separated and cultured in DMEM/F12 supplemented with 10% fetal calf serum (FCS) at 37°C, 5% CO_2 in air for 3-4 days followed by another 3-4 days under the same conditions but with 0.5% FCS (serum starvation culture). Percentages of cells in the various stages of the cell cycle were calculated using flow cytometry with gates were set to include only small, medium or large-sized cells. Gating for different cell sizes was guided by microscopic measurement of trypsinised cell population. Cells of different designated sizes were electrically fused to metaphase II arrested enucleated oocytes. Reconstituted embryos were cultured in modified synthetic oviduct fluid medium supplemented with amino acids, glucose, insulin and BSA at 39°C, 5% CO_2, 5% O_2 and 90% N_2. Development to blastocysts and cell count were checked 174 h post-fusion. Flow cytometric analysis revealed that small and medium-sized cells had significantly higher percentages ($p< 0.05$) of nuclei existing in the G0/G1 phase (93-94%) than large cells (83%). Serum-starved cells of different sizes fused at the same rate to the enucleated oocytes. No significant difference ($p>0.05$) was observed in cleavage, development to blastocyst and blastocysts cell number after embryos reconstruction from different cell sizes. In conclusion the present study showed that different sizes of the primary cultured, serum starved cumulus cells can be used as donor nuclei at the G/0G1-phase.

Key words: Cloning, nuclear transfer, serum starvation, cell cycle, cell culture

INTRODUCTION

An important factor controlling the developmental rates of reconstituted embryos is the appropriate cell cycle coordination of donor nuclei and recipient oocytes[1]. Nuclei transferred to metaphase II recipient cytoplasts during or before activation, when maturation-promoting factor levels are high must be in the G0/G1 stage of the cell cycle[1,2].

Serum starvation of cultured donor cells is the popular method to obtain cells in the G0/G1 phase of the cell cycle[2-4]. The production of live offspring after somatic cell nuclear transfer using fetal fibroblasts[2,5], adult mammary gland cells[2] and cumulus cells[4] was attributed to serum starvation of donor cells presumably induced to exit the cell cycle and enter the quiescent G0 phase prior to nuclear transfer[6], however non of these studies confirmed the cell cycle phase of the transferred donor nuclei. Therefore, this study concurrently investigates the cell cycle of the transferred nucleus.

For practical selection of cells at time of transfer, certain morphological criteria are to be associated with cells in the G0/G1 phase. It was reported that cellular volume increases from G1 through G2 + M phases of the cell cycle[7]. In this respect the size of donor cells would be a practical morphological criterion for selection of cells in the G0lG1 phase. Flow cytometric cycle analysis of porcine fetal fibroblast[8] and bovine cumulus cells [9] showed that as the cell size decreased, the percentages of cells existing in the G0 + G1 and G0 phases increased significantly. In view of this, one could imagine that the use of smaller donor cells would lead to higher embryonic

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development after nuclear transfer. However, small and large mouse cumulus cells that are naturally arrested in the G0 phase seldom support development of reconstituted embryos beyond 8-cell stage[10].

The present study was therefore, planned to concurrently investigate the cell cycle phase of the donor cells, correlate between cell size and cell cycle phae and the consequent development after nuclear transfer using primary cultured cumulus cells of different sizes.

MATERIALS AND METHODS

Recipient oocyte preparation: Cumulus-Oocyte Complexes (COCs) aspirated from small antral follicles of slaughter house ovaries were subjected to *in vitro* maturation as described elsewhere[11]. Briefly, the oocytes were cultured for 18 to 20 h in HEPES-buffered TCM 199 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS, Gibco), 0.02 units/ml FSH (from porcine pituitary, Sigma), 1 μg/mL estradiol 17ß (Sigma), 0.2 mM sodium pyruvate and 50 μg/mL gentamycin sulfate under a humidified atmosphere of 5% CO_2 in air at 39°C. Matured oocytes were enucleated at MII stage as described previously[12]. Enucleation was assessed by exposing oocytes under UV light for a few seconds.

Donor cells preparation: Cumulus cells collected 18 to 20 h after the start of maturation culture were separated with 0.1% hyaluronidase (Type 1-S, Sigma) dissolved in Ca^{2+}- and Mg^{2+}-free Dulbecco's PBS and washed several times in DMEM/F12 (Gibco). Cells were then cultured (8-9x10^4 live cells/ml) in DMEM/F12 (Gibco) supplemented with 10% FCS (Gibco) in 35x10 mm dishes (Falcon 3801, Becton Dickinson Labware, Franklin Lakes, NJ, USA) at 37°C, 5% CO_2 in under humidified air. Cultured cells were allowed to multiply for 3-4 days followed by another 3-4 days of culture in DMEM/F12+0.5% FCS to induce quiescence. After the designated culture period, the cells were disaggregated by trypsinization with Ca^{2+}- and Mg^{2+}-free Dulbecco's PBS containing 0.1% (w/v) trypsin (Sigma) and 0.1% (w/v) EDTA (Kanto Chemical Co., Tokyo, Japan) and used as donor nuclei.

Characterization of donor cells: Disaggregated cells from serum-starved cultures were characterized in terms of cell size, cell cycle phases of different cell sizes. Cell size was measured using the ocular scale under an inverted microscope (x400).

The cell cycle phase distribution was determined by measuring the DNA content of individual cells by flow cytometry as described previously[9]. Cells were washed with Ca^{2+}- and Mg^{2+}-free Dulbecco's PBS and then pelleted by centrifugation at 290 x g in a refrigerated centrifuge at 4°C. The pelleted cells were fixed in 70% ethanol and stored at-20°C. Just prior to flow cytometric analysis, individual samples were washed with Ca^{2+}- and Mg^{2+}-free Dulbecco's PBS and incubated with 200 μg/mL Rnase A (Boehringer Mannheim GmbH, Mannheim, Germany) dissolved in distilled water for 30 min at 37°C. Cells were then stained with 50 μg/mL propidium iodide (Sigma) dissolved in Ca^{2+}- and Mg^{2+}-free Dulbecco's PBS and were filtered through nylon mesh with 50 μm pores (Kyoshin Riko, Tokyo, Japan) immediately before analysis. Fluorescence obtained from 10^4 cells per sample was measured with an Epics XL-MCL flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA) using a 620-nm filter. The cell cycle distribution was determined using Epics XL system II software. The effect of cell size on the distribution of cells in the various phases of the cell cycle was determined using forward light scatter to separately gate on small, medium and large cells Fig. 1 and subsequent calculation of G0/G1, S and G2/M percentages within different gates. Gating for different cell sizes was achieved guided by the microscopically measured cell-size plotted histograms.

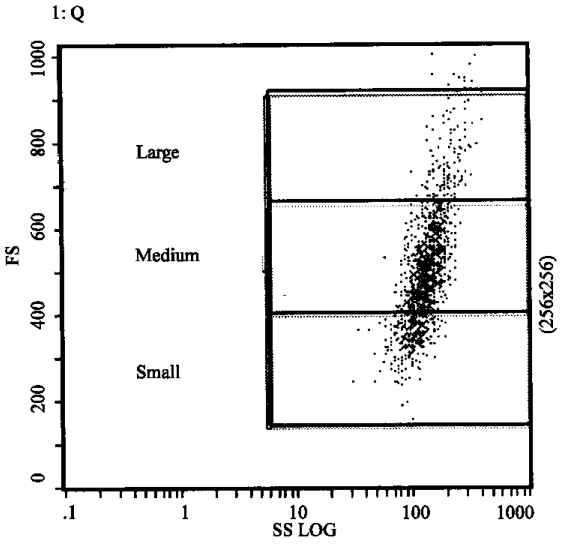

Fig. 1: Scatter plot of logarithmic side light scatter (SS LOG) versus forward light scatter (FS) of serum starved bovine cumulus cells allowing for gating to only include large, medium and small-sized viable cells

Production of nuclear transfer embryos: Trypsinized cumulus cells were inserted individually in the perivitelline space of the recipient cytoplast. Manipulated couplets were placed between two electrodes (0.5 mm apart), overlaid with 0.3 M mannitol containing 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$. Cell fusion was induced by 2 DC pulses of 0.9 kv/cm, for 40 μsec, 1 sec apart was delivered to the chamber using a BTX Electro Cell Manipulator 2001 M

(BTX, San Diego, CA, USA). Successfully fused couplets were incubated in the embryo culture medium supplemented with 10 μg/mL cycloheximide (Sigma) under a humidified atmosphere of 5% CO_2 in air at 39°C for 5-6 h. They were then thoroughly washed and subsequently cultured in the embryo culture medium (mSOFai) [11] supplemented with 1 mM glucose and 3 mg mL^{-1} fatty acid-free BSA (Sigma) instead of PVA under 5% CO_2, 5% O_2 and 90% N_2. The cleavage rate was determined at 33 h after fusion. Development to blastocysts and the cell count[13] were checked 174 h post-fusion.

Statistical analysis: Data were subjected to analysis of variance followed by Fisher's protected least significant difference test by using StatView (Abacus Concepts Inc., Berkeley, CA, USA). $p<0.05$ was considered to be statistically significant.

RESULTS

Cell size and cell cycle distribution: The cell line morphology of serum starved cultured cumulus cells and is shown in Fig. 2. The size distribution of these cells after trypsinization revealed that the majority were within the medium size (15-19 μm) range Fig. 3.

The flow cytometric histograms showing the DNA content of the total population of the serum starved cells are shown in Fig. 4. The computer analysis of small, medium and large sized cells is shown in Table 1. The percentages of nuclei existing in the G0/G1 phase for the small (94.4±2.7%) and medium cell populations (93.9±3.9%) were significantly higher ($p<0.05$) than those for large cells (83.3±13.7%). The percentages of cells in S and G2/M phases for large cells were significantly higher than those for small and medium-sized cells ($p<0.05$).

Table 1: Cell cycle distribution of cumulus cells after serum starvation culture

Cell size	Cell cycle phase (%)		
	G0/G1	S	G2/M
Small	94.4±2.7[a]	0.8±0.2[a]	0.5±0.1[a]
Medium	93.9±3.9[a]	1.0±1.0[a]	1.6±0.7[a]
Large	83.3±13.7[b]	3.3±1.6[b]	12.2±4.8[b]

% values are mean ± SD of 3 replicates, [ab] Values with different superscripts within a column differ significantly (at least $p<0.05$)

Table 2: Effect of the cell size of serum-starved cumulus cells on the development of nuclear transfer embryos

Cell size	No. of karyoplast cytoplast complexes	Fused (%)	Cleaved* (%)	Blastocyst* (%)	Blastocyst cell count (N)
Small (9-14 μm)	148	39.7±9.2	83.4±7.0	33.4±7.01	80.7±43.1 (15)
Medium (15-20 μm)	115	52.2±5.4	85.9±5.4	38.2±4.318	6.3±48.5(16)
Large (21-179.7±49.2(16)27 μm)	119	48.9±11.2	85.8±6.9	34.6±7.1	

% values are means ±SD of 4 replicates, *Based on the number fused

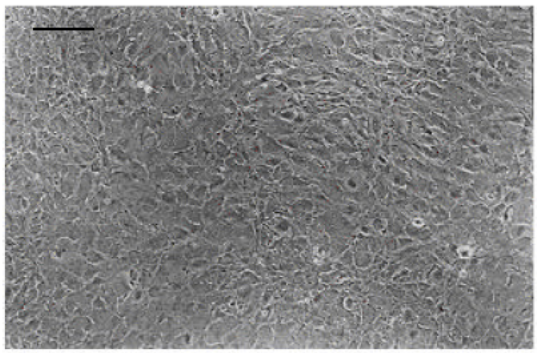

Fig. 2: Morphology of the cumulus cells after serum starvation culture. Bar = 20 μm

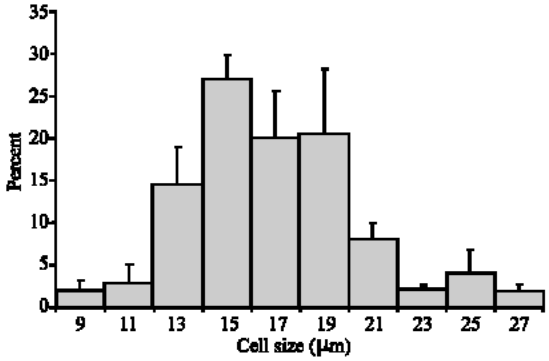

Fig. 3: Cell size distribution of cultured cumulus cells after serum starvation. Each bar represents mean ± SD of four replicates

Development of reconstituted embryos: As shown in Table 2, no significant difference was noticed in fusion rate for small, medium and large cells with recipient cytoplast ($p>0.5$) despite the higher tendency for the large sized cells. Embryos reconstituted from small, medium and large cells showed no significant difference in cleavage rate and in vitro development to the blastocyst stage Table 2.

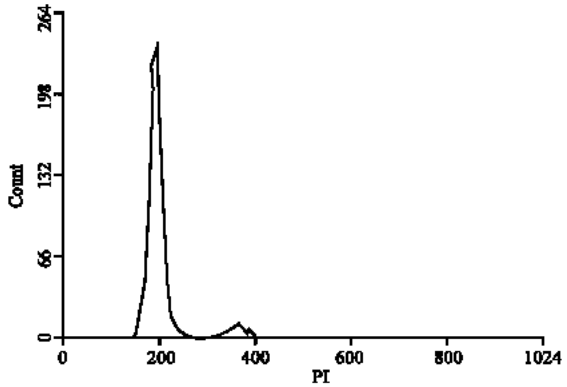

Fig. 4: Single parameter flow cytometry histogram of DNA content (PI) versus count for total cell population showing nuclei existing in the G0/G1, S and G2/M phases of the cell cycle after serum starvation culture

DISCUSSION

Nuclear transfer, a powerful technique for cloning and production of transgenic animal involves transferring a nucleus to an enucleated oocyte that was arrested at metaphase II of meiosis. For nuclear transfer to be successful, a factor of prime importance must be well thought-out. This factor is the coordination of the cell cycle phase of donor nucleus and recipient cytoplast[1]. When nuclei are transferred to M-phase cytoplasts in which maturation promoting factor activity is high, the nuclei undergo nuclear envelop break down, premature chromosome condensation and DNA replication after nuclear envelop reformation. Therefore, to maintain correct ploidy of nuclear transfer embryo, only G0- or G1-phase nuclei should be transferred to M-phase cytoplasts[1,14]. To bring cells in G0/G1 phase, cultured cells in this study were subjected to serum starvation[9,15-17]. Serum starvation conditions was reported to make cells exit the cell cycle and arrested in G0/G1 phase[2].

The somatic cell nuclear transfer in the previous nuclear transfer studies in bovine was not characterized at least in terms of morphology and cell cycle phase [3,18-21]. The present study concurrently investigated the donor cell cycle phase and their development after nuclear transfer.

Investigating the cell cycle phase of the serum starved cells, our result are in agreement with those of Boquest[8,22] for small and medium sized-cells of porcine fetal fibroblast and porcine mammary cells, respectively. In contrast, higher percentage of large sized cells were obtained in the G0/G1 in our study compared to previous studies[8,22], this difference could be due to the difference in species and type of cells as different cell population were reported to be affected differently by serum starvation[23].

The small and medium sized cells with the high percentage of nuclei in the G0/G1 phase exhibited the same developmental rate to the blastocyst when both sources of nuclei were used as donors. Despite the low percentage of G0/G1 nuclei of the large cells compared to small and medium sized ones, they resulted in the same in vitro development to blastocysts stage. Serum-starved large cells did not have significantly higher fusion rate than small and medium-sized cells as expected. This could have been because the surface contact between the recipient and the three cell sizes was the same. The fusion rate obtained in this study was lower than that obtained in previous study using cumulus cells[4]. This could be due to the difference in the experimental conditions.

CONCLUSION

The results of the present study indicate that the different cell sizes of cumulus cells under serum starvation culture condition have high percentage of cells in the G0/G1 phase of the cell cycle and can equally be used as nuclear donors in nuclear transfer procedure. The development to term using the small, medium and large size cell needs further investigation.

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