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FULL PAPER

Cell cycle analysis of bovine cultured somatic cells by flow cytometry

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Abstract

This study was undertaken to examine the cell cycle characteristics of bovine fetal and adult somatic cells (fetal fibroblasts, adult skin and muscle cells, and cumulus cells) after culture under a variety of conditions ; 1) growth to 60-70% confluency (cycling), 2) serum starvation, 3) culture to confluency. Cell-cycle phases were determined by flow cytometry with propidium iodide staining enabling the calculation of percentages of cells in G0/G1, S and G2/M. The majority was in G0/G1 regardless of cell type and treatment. Serum-starved or confluent cultures contained higher percentages of cells in G0/G1 (89.5-95.4% ; $P<0.05$). Percentages of cells in G0/G1 increased as cell size decreased regardless of the cell type and treatment. In the serum-starved and confluent cultures, about 98% of small cells were in G0/G1. Serum-starved cultures contained higher percentages of small cells (38.5-66.9%) than cycling and confluent cultures regardless of cell type ($P<0.05$). After trypsinization of fetal fibroblasts and adult skin cells that were serum-starved and cultured to confluency, the percentages of cells in G0/G1 increased ($P<0.05$) on incubation for 1.5 (95.7-99.5%) or 3 hr (95.9-98.6%). These results verify that serum starvation and culture to confluency are efficient means of synchronizing bovine somatic cells in G0/G1, and indicate that a more efficient synchronization of the cells in G0/G1 can be established by incubation for a limited time period after trypsinization of serum-starved or confluent cells.

Key words : bovine somatic cells ; cell cycle ; cell size ; flow cytometry

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Introduction

The cell cycle phase in which donor nuclei exist prior to nuclear transfer (NT) is an important factor governing developmental rates of reconstituted embryos. In mammalian embryonic cell NT, the nuclei transferred to inactivated metaphase II oocyte cytoplasm must be in the G1 phase of the cell cycle to maintain the ploidy of the reconstituted embryos[3,6]. Transfer of a G1 nucleus into an enucleated metaphase II (MII) oocyte facilitated nuclear reprogramming and led to higher development rates of reconstituted embryos[6,8]. In mammalian somatic cell NT, donor cells were synchronized to the quiescent G0 phase instead of G1[4,22]. The production of live offspring from adult and fetal somatic cells was reported to be possible when quiescent G0 cells were transferred into enucleated oocytes[14,20,22]. The quiescent G0 phase in somatic cells can be obtained by serum starvation[2,4,22] or by using cells naturally arrested at this stage directly from the animal, such as Sertoli or neuronal cells[19]. The G0 phase of the cell cycle is implicated in the reduction of transcriptional activity and chromatin modification[21], which may facilitate the reprogramming of the donor nucleus in the oocytes.

Some studies, however, suggested that the use of G0 cells might not be a critical factor for successful somatic cell cloning. Cibelli *et al*.[7] insisted that somatic cells in G1 could be successfully reprogrammed after NT. Nonquiescent proliferating cells, the majority of which were in G1[2,7], can support development of NT embryos to term[7,23]. Full-term development was also achieved with cumulus cells of a mouse, about 90% of which were in G0 / G1[19]. Cloned piglets were also produced from the NT of nonquiescent fetal fibroblasts[16].

Several methods have been employed to synchronize cells to G0 / G1. The most popular methods are serum starvation and culture to confluency[1,13,15]. Cell cycle analysis has also been made in some specific cell lines in pig and cattle[2,11,13,15,18]. However, in these studies, the cell cycle analysis was made only at the end of culture treatment. In a quest for optimized donor nucleus treatment prior to NT, this study was undertaken to examine the populations of bovine fetal and adult somatic cells existing various cell-cycle phases at the end of several culture treatments : cycling culture, serum starvation and culture to confluency. Furthermore, we investigated the changes in their populations with time after tripsinization. This was achieved by measuring the DNA content of cells using flow cytometry.

Materials and Methods

Preparation of somatic cells

Bovine fetal fibroblast cells were isolated from a male fetus (about 100 days old). The head, heart and intestine were removed from the fetus and the remaining body parts were chopped into small pieces. Adult skin- and muscle-derived fibroblast cells were obtained by ear biopsy of a 3-year-old Korean native cow. The tissues were cut into small pieces (1 mm^2) and were enzymatically digested with 0.05% trypsin-EDTA in Dulbecco's phosphate-buffered saline (DPBS ; Gibco-BRL, Grand Island, NY, USA) for 30 min at 37℃ with occasional stirring. The digestion procedure was repeated two times, and the digested tissues were allowed to settle for 5 min. The supernatants containing disaggregated cells were transferred into a 15-ml conical tube. Cells were collected by centrifugation at 500×g for 5 min and washed once in culture medium. The cell pellets were diluted with 3 ml of Dulbecco's modified Eagle's medium (DMEM ; Gibco-BRL) supplemented with 10% fetal bovine serum (FBS ; Gibco-BRL) and 50 μg/ml

gentamicin, and cultured in a 50-ml culture flask at 37℃ with 5 % CO_2 in air. Cumulus cells were isolated from cumulus-oocyte complexes by exposure to 0.1% hyarulonidase (Sigma) and were washed several times with DMEM. They were then cultured in 3 ml of DMEM supplemented with 10% FBS and 50 μg/ml gentamicin in a 50-ml culture flask. At about 90% confluency, the cells were passaged with a 1 : 2 division, and subsequently regrown cells were passaged four times more before being frozen in DMEM with 15% FBS and 10% dimethylsulfoxide and stored in liquid nitrogen.

Cell treatments

Thawed cells from each tissue were cultured to form a monolayer before use. Cells were cultured in DMEM supplemented with 10% FBS at a concentration of 1×10^5 cells/ml in ϕ35mm dishes (Nunculon, Kamstrup, Denmark). After 2 days of culture to 60-70% confluency, cells were allocated one of the following treatments before being fixed in ethanol : 1) immediate fixation (cycling cells); 2) replacement of medium with DMEM supplemented with 0.5% FBS and culture for 5 additional days (serum-starved cells); 3) change of medium every 2 - 3 days for an additional 2 weeks of culture (confluent cells).

Cell fixation and staining

Trypsinized cells were resuspended in DPBS and centrifuged at 500 × g for 5 min. Cell pellets were resuspended in 1 ml of 70% (v/v) ethanol (4 ℃) and fixed overnight, then kept at -20℃ until analysis. After ethanol fixation, cells were pelleted, then washed once with DPBS and incubated with 200 μg/ml RNase A (Boeringer Mannheim GmbH, Mannheim, Germany) at 37℃ for 30min. The cells were stained with 50 μg/ml propidium io-

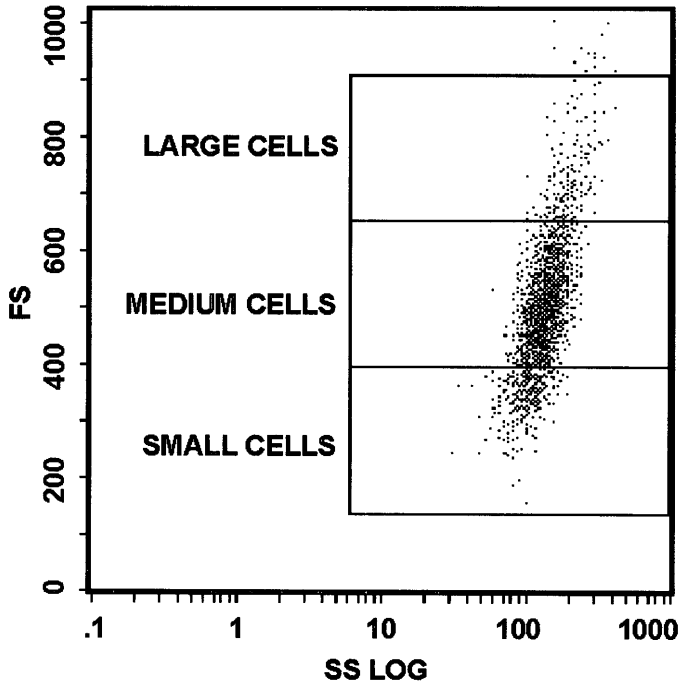

Fig. 1. Scatterplot of forward light scatter (FS) versus side light scatter (SS) of cycling bovine adult skin-derived fibroblast cells allowing for gating of the viable cell population.

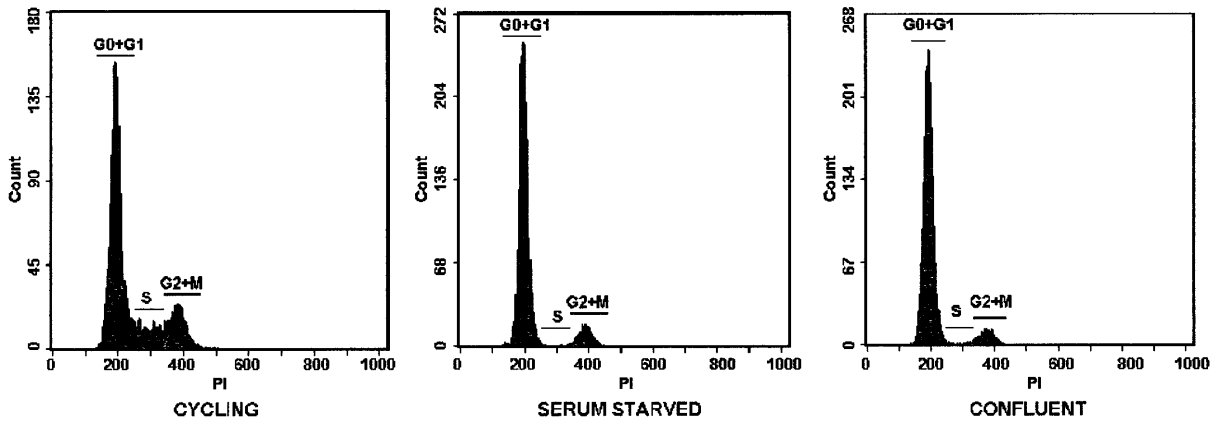

Fig. 2. Typical histograms of DNA obtained using flow cytometry of bovine adult skin-derived fibroblast cells following various culture treatments.

dide (Sigma) at room temperature for 1 hr. Stained cells were filtered through nylon mesh with 50-μm pores (Kyoshin Riko, Tokyo, Japan) just prior to flow cytometry.

Flow cytometry

Cell cycle phases were determined by measuring the DNA content of individual cells (1×10^4 cells) using flow cytometry (EPICS XL-MCL, Beckman Coulter Inc., FL, USA) as described previously[5]. The distribution of cells in various phases of the cell cycle was determined using forward scatter light to separately gate small, medium, and large cells (≤ 13, 14-18, and ≥ 19 μm, respectively; Fig. 1), and a subsequent calculation of percentages of cells in G0/G1, S and G2/M was made using Epics XL system II software (Fig. 2). Gating for different cell sizes was guided by the microscopically measured cell-size plotted histograms as described elsewhere[5].

Experimental design

Experiment 1 : The effects of culture conditions and cell size on the cell cycle were examined. Bovine fetal fibroblasts, adult skin- and muscle-derived fibroblasts, and cumulus cells were cultured under three sets of conditions; cycling culture, serum starvation and culture to confluency, and cell cycle phases were determined based on the culture conditions and cell size. The experiment was replicated three times (two samples for each treatment) on separate occasions.

Experiment 2 : The effect of incubation after the release from serum starvation or culture to confluency on the cell cycle was examined. Trypsinized fetal and adult skin fibroblast cells were resuspended with 1 ml of DMEM supplemented with 10% FBS in a 15-ml centrifuge tube and incubated at 37℃ for 1.5, 3, and 6 hr with occasional shaking before ethanol fixation to determine the cell cycle phase with the lapse of time after trypsinization of cells. The experiment was replicated three times (two samples for each treatment) on separate occasions.

Statistical analysis

Statistical analysis was performed using the General Linear Models procedure in the Statistical Analysis System (Cary, NC, USA). Differences between groups were determined using ANOVA followed by Duncan's multiple range test.

Table 1. Cell-cycle distribution of bovine somatic cells cultured under various conditions.

Cell type	Cell-cycle phase	Culture treatments		
		Cycling	Serum starvation	Confluency
Fetal fibroblast cells	G0/G1	73.3±0.9[a]	95.4±0.3[b]	95.4±0.3[b]
	S	15.0±0.9[a]	0.6±0.1[b]	1.4±0.1[b]
	G2/M	11.7±1.7[a]	4.0±0.3[b]	3.2±0.3[b]
Adult skin cells	G0/G1	75.3±0.7[a]	93.3±0.3[b]	90.3±2.2[c]
	S	7.2±0.5[a]	0.8±0.2[b]	2.0±0.5[c]
	G2/M	17.4±1.0[a]	6.2±0.5[b]	7.8±1.9[b]
Adult muscle cells	G0/G1	82.9±1.5[a]	89.5±0.9[b]	89.3±0.6[b]
	S	5.0±0.9[a]	0.8±0.2[b]	0.7±0.2[b]
	G2/M	12.1±0.7[a]	9.7±1.1[b]	10.0±0.6[b]
Cumulus cells	G0/G1	70.5±1.7[a]	91.1±0.4[b]	92.6±0.4[b]
	S	15.4±1.2[a]	1.1±0.2[b]	1.4±0.1[b]
	G2/M	14.1±0.5[a]	7.8±0.5[b]	6.1±0.3[b]

[a-c] Values (means±SD, n=6) with different superscripts in the same row differ significantly ($P<0.05$).

Table 2. Cell-size distribution of cultured bovine somatic cells.

Cell type	Cell size	Culture treatment		
		Cycling	Serum starvation	Confluency
Fetal fibroblast cells	Small	14.0±2.9[a]	57.2±1.2[b]	38.3±3.3[c]
	Medium	55.2±1.2[a]	39.3±1.1[b]	52.9±2.1[a]
	Large	30.8±4.1[a]	3.6±0.2[b]	8.8±1.3[c]
Adult skin cells	Small	25.0±2.2[a]	62.4±1.2[b]	34.1±0.8[c]
	Medium	57.5±0.8[a]	33.6±0.6[b]	52.7±0.4[c]
	Large	17.5±1.7[a]	4.0±0.7[b]	13.2±1.2[c]
Adult muscle cells	Small	23.4±2.2[a]	66.9±4.1[b]	46.5±4.0[c]
	Medium	62.1±0.5[a]	28.3±3.2[b]	48.3±3.2[c]
	Large	14.6±1.8[a]	4.7±0.8[b]	5.2±0.9[b]
Cumulus cells	Small	10.5±2.8[a]	38.5±3.5[b]	25.3±1.2[c]
	Medium	60.5±0.6[a]	53.3±2.6[b]	60.6±0.7[a]
	Large	28.9±2.2[a]	8.2±1.0[b]	14.1±0.6[c]

[a-c] Values (means±SD, n=6) with different superscripts in the same row differ significantly ($P<0.05$).

Results

Experiment 1

Based on the DNA content histograms (Fig. 2), the majority of cells were in the G0/G1 phase regardless of the cell type and culture treatment (Table 1). Compared to values in cycling cells, however, significantly higher percentages of cells in G0/G1 were apparent when cells were serum-starved (89.5-95.4%) or cultured to confluency (89.3-95.4%) regardless of cell type ($P<0.05$). Conversely, cycling cells contained greater percentages of cells in the S (5.0-15.4%) and G2/M phases (11.7-17.4%) than other treatments regardless of cell type ($P<0.05$). In all cell types except skin cell, there were no differences in cell-cycle phase distribution between-serum starved and confluent cells. In the skin cells, the serum-starved group contained a higher percentage of cells in G0/G1 than the confluent group ($P<0.05$).

Microscopic measurement of a population of trypsinized cells showed that cell diameters ranged between 8 and 24 μm, and that the starved culture contained higher percentages

Table 3. Cell-cycle distribution of cultured bovine somatic cells in relation to cell size.

Cell type	Cell-cycle phase	Culture treatment and cell size								
		Cycling			Serum starvation			Confluency		
		Small	Medium	Large	Small	Medium	Large	Small	Medium	Large
Fetal fibroblast cells	G0/G1	89.4±2.1[a]	71.9±0.7[b]	68.9±1.1[c]	98.4±0.1[a]	90.6±1.9[b]	64.4±9.4[c]	99.3±0.1[a]	95.3±0.6[b]	79.0±2.6[c]
	S	8.4±1.9[a]	17.0±1.7[b]	14.2±0.7[b]	0.3±0.0[a]	2.3±0.4[b]	5.0±1.5[c]	0.5±0.1[a]	1.6±0.2[b]	4.6±0.7[c]
	G2/M	2.2±0.7[a]	11.1±2.3[b]	16.9±1.1[c]	1.3±0.1[a]	7.2±1.8[b]	30.6±9.1[c]	0.3±0.0[a]	3.2±0.5[b]	16.5±1.9[c]
Adult skin cells	G0/G1	93.3±0.9[a]	78.0±0.9[b]	40.4±0.9[c]	98.9±0.2[a]	87.9±1.4[b]	41.4±1.2[c]	98.8±0.7[a]	91.4±1.7[b]	65.4±4.3[c]
	S	2.6±0.1[a]	8.5±0.5[b]	9.8±1.2[b]	0.4±0.1[a]	1.4±0.3[b]	5.3±1.0[c]	0.5±0.1[a]	1.8±0.1[b]	4.7±0.3[c]
	G2/M	4.1±1.0[a]	13.5±1.0[b]	49.7±1.6[c]	0.8±0.2[a]	10.6±1.3[b]	53.3±2.1[c]	0.8±0.6[a]	6.8±1.6[b]	29.9±4.1[c]
Adult muscle cells	G0/G1	96.9±0.4[a]	85.6±1.0[b]	48.7±1.6[c]	98.1±0.4[a]	79.8±0.7[b]	27.1±2.3[c]	97.7±0.5[a]	79.5±4.4[b]	32.4±4.6[c]
	S	1.1±0.1[a]	5.7±1.0[b]	8.2±0.9[c]	0.3±0.2[a]	1.2±0.1[b]	2.9±1.0[b]	0.5±0.2[a]	0.9±0.3[ab]	1.8±0.4[b]
	G2/M	2.0±0.3[a]	8.6±0.2[b]	43.1±1.0[c]	1.6±0.2[a]	19.0±0.6[b]	70.0±3.1[c]	1.8±0.4[a]	19.6±4.2[b]	65.8±4.3[c]
Cumulus cells	G0/G1	94.8±0.9[a]	77.2±1.3[b]	46.4±3.1[c]	98.9±0.4[a]	91.2±1.2[b]	48.7±2.1[c]	99.1±0.1[a]	95.1±0.4[b]	70.6±1.3[c]
	S	4.2±0.7[a]	14.7±2.1[b]	18.7±2.1[b]	0.4±0.1[a]	1.1±0.1[b]	2.7±0.9[b]	0.5±0.0[a]	1.4±0.1[b]	2.3±0.4[b]
	G2/M	1.0±0.8[a]	8.1±1.6[b]	34.9±3.9[c]	0.8±0.3[a]	7.7±1.2[b]	48.6±2.7[c]	0.4±0.1[a]	3.5±0.4[b]	27.1±1.0[c]

[a-c] Values (means±SD, n= 6) with different superscripts within the same row for each treatment differ significantly ($P<0.05$).

Table 4. Cell-cycle distribution of incubated bovine fetal-and adult skin-derived fibroblasts after release from serum starvation or confluency.

Cell type	Culture treatment	Cell cycle phase	Time (hr) after trypsinization			
			0	1.5	3	6
Fetal fibroblast cells	Serum starvation	G0+G1	95.6±0.8[a]	99.5±0.1[b]	98.6±0.3[bc]	97.5±0.2[ac]
		S	0.7±0.2[a]	0.3±0.1[b]	0.5±0.1[ab]	0.8±0.3[ab]
		G2+M	3.8±0.6[a]	0.2±0.1[b]	0.9±0.3[b]	1.7±0.3[c]
	Confluency	G0+G1	95.1±0.5[a]	99.0±0.1[b]	98.3±0.2[b]	89.9±0.9[c]
		S	1.5±0.3[a]	0.5±0.1[b]	0.7±0.1[b]	5.1±1.2[c]
		G2+M	3.3±0.3[a]	0.5±0.1[b]	1.1±0.2[b]	4.9±0.4[c]
Adult skin cells	Serum starvation	G0+G1	93.3±0.5[a]	96.4±0.5[b]	96.0±0.5[b]	94.2±0.1[a]
		S	0.8±0.1	0.7±0.2	0.7±0.2	0.7±0.1
		G2+M	5.9±0.4[a]	2.8±0.6[b]	3.3±0.6[b]	5.1±0.1[a]
	Confluency	G0+G1	92.0±1.2[a]	95.7±0.8[b]	95.9±0.3[b]	90.5±0.7[a]
		S	2.0±0.2[a]	1.0±0.3[b]	0.6±0.2[b]	2.2±0.2[a]
		G2+M	6.0±1.3[a]	3.3±0.5[b]	3.5±0.3[b]	7.3±0.9[a]

[a-c] Values (means±SD, n= 6) with different superscripts in the same row differ significantly ($P<0.05$).

of small cells (38.5-66.9%) than cycling (10.5-25.0%) and confluent cultures (25.3-46.5%) regardless of cell type (Table 2 ; $P<0.05$). The percentages of small cells were higher in the confluent culture than cycling culture ($P<0.05$). Regardless of the cell type and treatment, percentages of cells in G0/G1 increased ($P<0.05$), whereas percentages of S- and G2/M-phase cells decreased as cell size decreased from large to small (Table 3).

Experiment 2

After trypsinization of fetal fibroblast and adult skin cells that were serum starved and cultured to confluency, the percentages of cells in G0/G1 increased ($P<0.05$) with time to 1.5 (95.7-99.5%) and 3 hr (95.9-98.6%), then decreased to similar levels as those in the initial cell cycle phase (at time of trypsinization ; 0 hr) by 6 hr (Table 4).

Discussion

When somatic nuclei are transferred to metaphase II oocyte cytoplasts, it is generally accepted that the donor nucleus must be in the G0 or G1 phase, to maintain the ploidy of the reconstituted embryos at the end of the first cell cycle[3,4,6]. However, it is not clear whether G0 or G1 is more appropriate. In somatic cell NT, Campbell *et al*.[4] and Wilmut *et al*.[22] stated that the nucleus must be in G0, while Cibelli *et al*.[7] and Lai *et al*.[16] showed that cycling cells or non-cultured cells immediately after thawing (presumably G1 cells) can be used as nuclear donor cells.

In previous cell cycle studies of pig fetal fibroblasts[2] and mammary cells[18], the population of G0 cells was calculated based on the protein content by staining with fluorescein isothiocyanate. Cells in G0, in quiescence, would be expected to contain much less RNA and protein[22]. The acridine orange staining method can also discriminate between G0 and G1 by quantification of RNA and DNA[9]. In the present study, however, we estimated the population of cells in G0/G1, S and G2/M based on the DNA content. We did not make a discrimination between G0 and G1 due to the limitations of flow cytometric methods based on propidium iodide staining[9].

Serum starvation and culture to confluency were valuable methods to synchronize the cells in G0/G1 regardless of the cell type, although it was suggested that serum starvation could induce cell death via apoptosis[12,17]. Similar results were obtained from bovine ear skin fibroblasts[15], pig cumulus cells[5], pig fetal fibroblast cells[2] and pig mammary cells[18] after serum starvation or culture to confluency. Cell cycle distributions of cycling cells were also similar to previous studies in bovine[15] and pig[2,18], in which 65-75% of cycling cells were in G0/G1. In the present study, more than 70% of cycling cells were in G0/G1 regardless of the cell type, and more than 90% of small cycling cells were in G0/G1. One can therefore predict that the use of serum-starved or confluent cells, and even small-sized cycling cells will result in greater development of reconstituted embryos.

In the somatic cell NT system, trypsinized donor cells are usually placed in culture before and during NT. During this time, donor cells can stabilize, form a round shape and progress through their cell cycle. The population of cells in G0/G1 increased with culture for 1.5 to 3 hr after trypsinization of serum-starved and confluent cells. It seems that while cells in the late G2 and M phases at the time of trypsinization divided and entered the G1 phase, those in G1 stayed at their initial stages during culture for 1.5 or 3 hr. It was suggested that mammalian somatic cells have an inherently long G1 phase[10,18]. Long-term culture for up to 6 hr, however, resulted in a re-increase in the percentages of cells in S and G2/M, which suggests that some cells in G1 and S transited to S and G2, respectively.

In conclusion, the results of this study provide information on the optimal treatment of donor cells for NT in relation to cell cycle synchronization. The results verify that serum starvation and culture to confluency are efficient for the synchronization of bovine somatic cells in G0/G1. They also indicate that a more efficient synchronization of the cells in G0/G1 for NT can be established by incubation for a limited period of time after trypsinization of serum-starved or confluent cells.

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