

Short Communication

Flow cytometric cell cycle analysis of cultured brown bear fibroblast cells

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Abstract

The aim of this study was to assess by flow cytometry the cell cycle of brown bear fibroblast cells cultured under different growth conditions. Skin biopsies were taken in Cantabria (Spain) from a live, anaesthetized brown bear. DNA analysis was performed by flow cytometry following cell DNA staining with propidium iodide. Serum starvation increased ($P < 0.01$) the percentage of G0/G1 phase cells (92.7 ± 0.86) as compared to cycling cells (39.7 ± 0.86) or cells cultured to confluency (87.3 ± 0.86). DMSO included for 48 h in the culture significantly increased ($P < 0.01$) the percentage of G0/G1 phase of the cell cycle at all concentrations used and decreased percentages of S phase in a dose-dependent fashion. Roscovitine increased the G0/G1 phase of the cell cycle ($P < 0.01$) at 15 μM concentration. Interestingly, the G2/M stage significantly increased at 30 and 50 μM compared to the control and 15 μM ($P < 0.02$). The cell cycle of brown bear adult fibroblast cells can be successfully synchronized under a variety of culture conditions.

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1. Introduction

Assisted reproductive technologies have been used to help in the preservation of endangered or threatened animals such as the African wild cat (Gomez et al., 2003) and giant panda (Han et al., 2003; Spindler et al., 2004; Hori et al., 2006). Although nuclear transfer raises controversial questions in its applications to wildlife conservation (Holt et al., 2004), the potential of this technology as a valuable tool for aiding in

the conservation of some endangered and threatened animals should not be ignored. In Spain, the Cantabric brown bear (*Ursus arctos pyrenaicus*) is at risk of extinction with a population estimated at only 100 animals. Efforts have been made to protect the declining environmental conditions and to preserve semen, cells and somatic tissues.

When populations or sub-population are at risk of extinction, nuclear transfer may be a valuable approach for species restoration (Gomez et al., 2006). Somatic cell nuclear transfer (SCNT) has been successfully applied in domestic and laboratory animals and in wild animals (Gomez et al., 2003; Loi et al., 2001; Williams et al., 2006). The control of cell cycle stage of donor cells is a relevant factor in the development of SCNT embryos. Differences in DNA content of donor nuclei vary according to the phase of the cell cycle and may affect the interaction with the recipient cytoplasts. Researchers have used different approaches to synchronize the cell cycle of the donor cells, among them, cell confluency-contact inhibition

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(Hinrichs et al., 2006) and serum starvation (Li et al., 2003). In addition, chemical inhibitors have been used such as roscovitine (Gibbons et al., 2002), dimethyl sulfoxide (DMSO) (Hashem et al., 2007), butyrolactone I (Kues et al., 2000), aphidicolin (Collas et al., 1992), demecolcine (Li et al., 2005), Hoechst 33342 (Kühholzer and Prather, 2001), mimosine (Vacková et al., 2003) or colchicine (Lai et al., 2001) that result in cell cycle arrest at specific points. However, no work has been done on the control of the cell cycle stages in brown bear. The aim of this study was to assess by flow cytometry the cell cycle of brown bear fibroblast cells cultured under a variety of cell cycle-arresting treatments.

2. Materials and methods

2.1. Establishment and culture of fibroblast cells

Skin biopsies were taken in Cantabria (Spain) from a live, anaesthetized brown bear (*Ursus arctos*). A procedure to obtain culture and cryopreserve skin-derived fibroblasts from brown bears has been described (Caamaño et al., 2005). Briefly, two skin biopsies were taken from the inner thigh. Biopsies were manually cut into small pieces, mixed together and enzymatically digested with collagenase Type IV (300 units/mL) (Sigma C5138) for 14 h at 38 °C. Disaggregated cells were centrifuged at 1600 rpm for 10 min and the pellet was diluted with D-MEM (Sigma D5671) containing 10% Fetal Bovine Serum (FBS). Cells were placed in a 25 cm² flask for culture under 5% CO₂ in air and high humidity at 38 °C. Confluent fibroblast monolayer was obtained after five days in culture. Two to four passages were performed using 75 cm² flasks before freezing fibroblasts in D-MEM containing 10% DMSO and 10% FBS.

2.2. Flow cytometric analysis

DNA content and cell cycle analysis were performed by flow cytometry. Cell suspensions and DNA staining with propidium iodide were performed following an optimized method based on the Vindelöv technique (Vindelov et al., 1983). Samples were analyzed in a Cytomics FC-500 cytometer (Beckman Coulter). A total of 20,000 cells per sample were collected by using a 488 nm excitation and a 605–635 nm bandpass filter. Cells were gated on forward light scatter versus side light scatter such that only cells without debris were assayed. The DNA histogram analysis was performed prior to manual elimination of aggregates by Modfit LT 3.0 software (Verity Software House), and the percentages of cells existing within the various phases of the cell cycle were automatically calculated by the program with the same algorithm in all the samples.

2.3. Cell treatments

In experiment 1, thawed fibroblast cells (second passage) were seeded in three 25 cm² flasks (8.5 × 10⁵ cells/flask). After 24 h in culture, fibroblast cells were exposed to one of

three treatments: (1) cells cultured to 70–80% confluency (cycling cells), (2) cells cultured to 100% confluency and then cultured for an additional five days (contact inhibition) or (3) cells cultured in serum-starved conditions for five days.

Fibroblast cells were exposed to cell cycle inhibitors and dose–response experiments were performed for each of the chemicals, DMSO and roscovitine. In experiment 2, cells were seeded in four 75 cm² flasks at a concentration of 1.25 × 10⁶ cells/flask and cultured for 24 h. The culture medium was removed and replaced with medium containing DMSO (Sigma D5879) at 0%, 1%, 2% or 3% for 48 h. In experiment 3, thawed fibroblast cells were seeded in four 75 cm² flasks at a concentration of 1 × 10⁶ cells/flask and cultured for 24 h. The culture medium was removed and replaced with culture medium containing roscovitine (Sigma R7772) at a concentration of 0, 15, 30, or 50 μM and cultured for another 24 h. In all the above experiments, cells were cultured under the same conditions (5% CO₂ in air and high humidity at 38 °C).

2.4. Statistical analyses

The above experiments were replicated three times. In each experiment, cells from each replicate were analyzed by flow cytometry on separate occasions. On each occasion, two samples of each treatment were analyzed ($n = 6$). Statistical analysis was performed by using the GLM procedure of SAS. Differences between treatments were determined by using LSM and were considered significant when $P < 0.05$.

3. Results

In experiment 1, the percentages of G0/G1 and S phase cells differed under different growth conditions. Serum starvation for five days increased ($P < 0.01$) the percentage of G0/G1 phase cells as compared to cycling cells or cells cultured to confluency. Cells cultured to 70–80% confluency contained higher ($P < 0.01$) percentages of S and G2 + M cells compared to cells cultured to confluency or serum starved, respectively (Table 1 and Fig. 1).

Cell cycle inhibitors, DMSO and roscovitine, were added to the cultures in an attempt to synchronize adult fibroblast cells in G0/G1 phase of the cell cycle. In experiment 2, DMSO included for 48 h in culture significantly increased ($P < 0.01$) the percentage of cells in G0/G1 phase at all concentrations used. However, only DMSO at 3% showed an

Table 1
Percentages (±SD) of brown bear fibroblasts existing in the various phases of the cell cycle after treatment with different growth conditions

Treatment	Cell cycle phase		
	G0/G1	S	G2/M
70% Confluency	39.6 ± 3.4 ^a	35.1 ± 2.3 ^a	25.2 ± 4.4 ^a
100% Confluency (contact inhibition)	87.2 ± 1.0 ^b	8.3 ± 2.1 ^b	4.4 ± 1.1 ^b
Serum-starved	92.7 ± 0.9 ^c	1.7 ± 0.4 ^c	5.5 ± 0.8 ^b

^{a,b,c} Values within a column having unlike superscripts are different ($P < 0.01$).

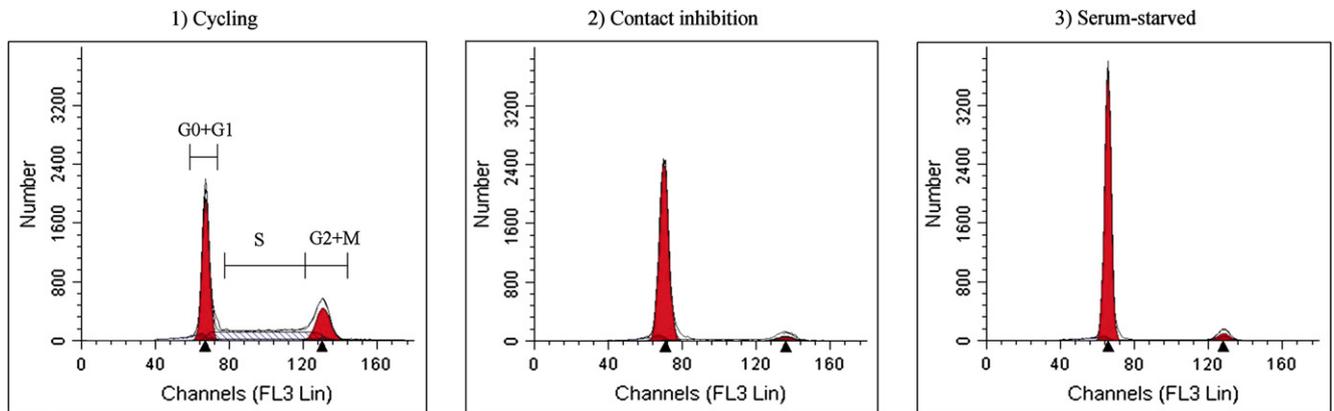


Fig. 1. Typical histograms of DNA obtained using flow cytometry of brown bear fibroblast cells cultured under different growth conditions.

increased percentage of cells in G2 stage ($P < 0.01$). DMSO decreased the percentages of S phase in a dose-dependent fashion (Table 2). Fig. 2 shows fibroblast cells cultured with 0%, 1%, 2% or 3% DMSO for 48 h. DMSO at higher concentrations exerted a negative effect on total cell numbers (data not shown).

When roscovitine was added to the cultures in a dose–response experiment, only 15 μM significantly increased the percentage of cells in G0/G1 phase ($P < 0.01$), while the percentage of cells in S phase decreased at all concentrations used in this experiment compared to the control group ($P < 0.01$). Interestingly, roscovitine (30 and 50 μM) significantly increased the proportions of cells in G2/M stage compared to 15 μM and the control (Table 3).

4. Discussion

In this study, flow cytometry has allowed us to analyze the cell cycle characteristic of brown bear adult fibroblast cells under a variety of cell cycle-arresting treatments. Serum starvation and induction of a confluent monolayer elicited a higher percentage of cells in G0/G1 and a lower percentage of S and G2/M stages than cycling cells. When taken in context of differences in cell types and animal species, the present results are in agreement with those of Gomez et al. (2003) in African wild cat and domestic cat, Boquest et al. (1999) in pig, Hayes et al. (2005) in cattle, Saikhun et al. (2004) in swamp buffalo, Han et al. (2003) in giant panda, Li et al. (2003) in ferret and Liu et al. (2004) in rabbit. Cultured adult fibroblasts from a Siberian tiger (Hashem et al., 2007) showed

a similar trend in relation to G0/G1. However, the S and G2/M stages did not differ significantly in the cycling cells, serum-starved cells and confluent monolayer. In contrast, goral adult fibroblast cells did not show differences in the proportion of G0/G1 between treatments (Hashem et al., 2006). Moreover, a fully confluent monolayer produced higher proportion of S and G2/M stages than serum starved and cycling cells. Differences in the results could be explained by differences in experimental protocols, animal species, cell type and number of passages used in the culture of fibroblasts.

Alternatively, cells can be arrested in the cell cycle by exposure to reversible cell cycle inhibitors such as DMSO and roscovitine. In this study, DMSO included for 48 h in culture significantly increased the percentage of cells in G0/G1 phase, while the percentage of cells in S stage decreased in a dose-dependent fashion. DMSO did not affect the cell cycle stages of cultured skin fibroblasts of the Siberian tiger (Hashem et al., 2007). Culture of goral skin fibroblast with 0.5% and 1% DMSO for 24 h showed an increased percentage of cells in the G0/G1 stage (Hashem et al., 2006). However, 2.5% DMSO used for either 4 or 24 h also has an effect on goral fibroblast cells, decreasing the percentage in G0/G1 and the G2/M stages. These apparently contradictory results may depend on concentration of DMSO used or species sensitivity to this chemical. Roscovitine has been effective in arresting the cell cycle in mammals (Mermillod et al., 2000). In bovine, Gibbons et al. (2002) showed that roscovitine improved cell cycle synchrony and the nuclear reprogramming capacity of granulosa cells at G0/G1 stage, resulting in enhanced fetal and calf survival and increased cloning efficiency. Roscovitine did not affect the proportion of cells in G0/G1 stage in horse (Hinrichs et al., 2006), while in African wild cats and domestic cats, a higher percentage of adult fibroblast cell nuclei was in the G0/G1 phase after roscovitine treatment (Gomez et al., 2003). In our study, roscovitine at lower concentration (15 μM) increased the percentage of cells in G0/G1 phase, while the percentage of cells in S phase decreased at all concentration used. In addition, the percentage of cells in G2/M stage increased with 30 μM and 50 μM roscovitine. One possible explanation of the effects of roscovitine showed under our experimental conditions is that roscovitine could have a preferential

Table 2
Percentage (\pm SD) of brown bear fibroblast cells existing in the various phases of the cell cycle after treatment with different levels of DMSO for 48 h

DMSO (%)	Cell cycle phase		
	G0/G1	S	G2/M
0	71.0 \pm 0.9 ^a	17.8 \pm 1.3 ^b	11.1 \pm 0.4 ^{ab}
1	79.1 \pm 0.8 ^b	10.6 \pm 0.8 ^b	10.2 \pm 0.3 ^a
2	79.1 \pm 1.1 ^b	9.1 \pm 0.4 ^b	11.7 \pm 0.9 ^b
3	79.0 \pm 2.1 ^b	5.2 \pm 1.6 ^c	15.6 \pm 0.8 ^c

^{a,b,c} Values within a column having unlike superscripts are different ($P < 0.01$).

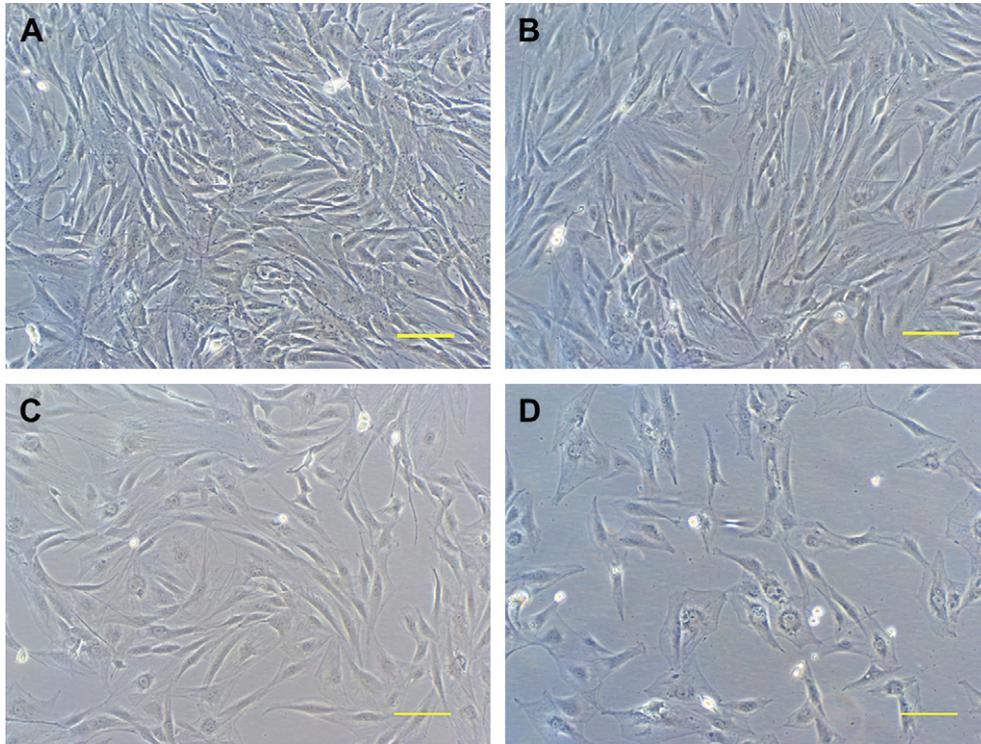


Fig. 2. Brown bear fibroblast cells cultured with 0 (A), 1 (B), 2 (C), or 3 (D) percentage of DMSO for 48 h (100 \times). Scale bar = 100 μ m.

inhibition on a specific cyclin-dependent kinase (CDK) at lower concentrations, while it would need higher concentrations to affect other CDKs, such as observed with human fibroblasts (Alessi et al., 1998).

When a metaphase II oocyte with a high level of maturation promoting factor is used as a recipient ooplasm, a G0/G1 or pre-S phase donor nucleus is required (Campbell et al., 1996). Further research will be necessary and interspecies SCNT should be considered to assess nuclear reprogramming with brown bear fibroblasts. A careful evaluation of the outcome of using brown bear fibroblast cells exposed to chemical and non-chemical treatments for interspecies SCNT would be necessary before reaching a definite conclusion on which treatment would be the most adequate for embryo production. Several authors reported that serum starvation induced higher rates of DNA fragmentation (Boquest et al., 1999; Kues et al., 2000; Gibbons et al., 2002) which could lead to failure in nuclear reprogramming after NT. Some of the chemical treatments could also be detrimental to the cells such as DMSO in our experiment. DMSO seemed detrimental for brown bear

fibroblast cells at higher concentrations (2% and 3%). These results are not surprising, since DMSO could be toxic for the cells.

In conclusion, we used flow cytometry to assess the cell cycle characteristics of brown bear adult fibroblasts. The results of the present study indicate that the cell cycle of brown bear adult fibroblast cells can be successfully synchronized under a variety of culture conditions. Serum starvation, confluent monolayer, 1% DMSO and 15 μ M roscovitine have been shown to be effective in synchronizing brown bear fibroblasts in G0/G1 stage of the cell cycle.

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Table 3

Percentage (\pm SD) of brown bear fibroblast cells existing in the various phases of the cell cycle after treatment with roscovitine for 24 h

Roscovitine (μ M)	Cell cycle phase		
	G0/G1	S	G2/M
0	62.4 \pm 3.0 ^a	24.8 \pm 1.5 ^a	12.7 \pm 2.0 ^a
15	76.4 \pm 5.9 ^b	8.5 \pm 1.9 ^b	15.1 \pm 4.6 ^a
30	64.5 \pm 3.3 ^a	11.1 \pm 0.7 ^c	24.3 \pm 2.7 ^b
50	67.1 \pm 3.4 ^a	9.5 \pm 0.7 ^{bc}	23.3 \pm 2.8 ^b

^{a,b,c} Values within a column having unlike superscripts are different ($P < 0.02$).

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