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Effect of serum starvation and chemical inhibitors on cell cycle synchronization of canine dermal fibroblasts

R. Khammanit [a], S. Chantakru [a], Y. Kitiyanant [b,c], J. Saikhun [b,*]

[a] Department of Anatomy, Faculty of Veterinary Medicine, Kasetsart University, Bangkok 10900, Thailand
[b] Institute of Science and Technology for Research and Development, Mahidol University, Salaya, Nakhonpathom 73170, Thailand
[c] Department of Anatomy, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

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Abstract

The cell cycle stage of donor cells and the method of cell cycle synchronization are important factors influencing the success of somatic cell nuclear transfer. In this study, we examined the effects of serum starvation, culture to confluence, and treatment with chemical inhibitors (roscovitine, aphidicolin, and colchicine) on cell cycle characteristics of canine dermal fibroblast cells. The effect of the various methods of cell cycle synchronization was determined by flow cytometry. Short periods of serum starvation (24–72 h) increased ($P < 0.05$) the proportion of cells at the G0/G1 phase (88.4–90.9%) as compared to the control group (73.6%). A similar increase in the percentage of G0/G1 ($P < 0.05$) cells were obtained in the culture to confluency group (91.8%). Treatment with various concentrations of roscovitine did not increase the proportion of G0/G1 cells; conversely, at concentrations of 30 and 45 μM, it increased ($P < 0.05$) the percentage of cells that underwent apoptosis. The use of aphidicolin led to increase percentages of cells at the S phase in a dose-dependent manner, without increasing apoptosis. Colchicine, at a concentration of 0.1 μg/mL, increased the proportion of cells at the G2/M phase (38.5%, $P < 0.05$); conversely, it decreased the proportions of G0/G1 cells (51.4%, $P < 0.05$). Concentrations of colchicines >0.1 μg/mL did not increase the percentage of G2/M phase cells. The effects of chemical inhibitors were fully reversible; their removal led to a rapid progression in the cell cycle. In conclusion, canine dermal fibroblasts were effectively synchronized at various stages of the cell cycle, which could have benefits for somatic cell nuclear transfer in this species.

Keywords: Cell cycle stage; Cell cycle synchronization; Dermal fibroblast; Somatic cell nuclear transfer; Dog

1. Introduction

Somatic cell nuclear transfer (SCNT) has been successfully used to produce cloned offspring in a number of mammalian species. However, the efficiency of cloning in all species has been extremely low. The first cloned dog was obtained by SCNT using ear skin fibroblasts for donor nuclei [1]. The development of reconstructed embryos following SCNT appeared to be dependent upon a variety of factors, including cell cycle synchronization between the donor nucleus and recipient oocyte [2]. It is generally accepted that when a metaphase II (MII) oocyte with a high level of maturation promoting factor (MPF) was used as recipient ooplasm, a G0/G1 or pre-S phase donor nucleus were required [3–6]. In contrast, when pre-activated oocytes were used as recipient cytoplast, both G- and S-phase donor nuclei were acceptable for NT. Moreover, the use of G2/M-stage

* Corresponding author. Tel.: +662 4419003–7x1389–90; fax: +662 4411013.
E-mail addresses: stjsk@mahidol.ac.th, jsaikhun@yahoo.com (J. Saikhun).

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donor cells can result in production of viable-cloned piglets with normal ploidy [7].

The cell cycle stages of cultured cells can be synchronized by serum starvation, contact inhibition, and chemical treatments. Serum starvation is widely used for synchronizing donor cells by arresting them in the G0/G1 phase of the cell cycle, but it often reduced cell survival and increased DNA fragmentation [8], which caused high-embryonic losses after NT [9]. It has been reported that roscovitine, a specific cyclin-dependent kinase (CDK) 2 inhibitor more efficiently synchronized bovine granulosa cells in the G0/G1 phase of the cell cycle than serum starvation, and resulted in an increase in cloning efficiency as defined in terms of survival of NT-derived fetuses and calves following embryo transfer [10]. Aphidicolin is a reversible inhibitor of mammalian DNA polymerases that blocks the cell cycle at the transition from G1 to S phase [11]. It has been shown that rodent and porcine fibroblasts can be reversibly synchronized at the S phase with aphidicolin treatment [8,12]. To synchronize cultured cells in the G2/M phase of the cell cycle, colchicine (a microtubule inhibitor) has been used to increase proportions of G2/M cells from 13 to 27-32% in pig mammary cells, and up to 37% in fibroblasts [13]. Although procedures for cell cycle synchronization in many mammalian species have been described, information on regulation of cell cycle stages in canine fibroblasts is extremely rare. Therefore, the objective of the present study was to investigate the efficacy of using serum starvation, culture to confluence, roscovitine, aphidicolin, and colchicine, to regulate the cell cycle stages of cultured canine dermal fibroblasts.

2. Materials and methods

2.1. Chemicals

All chemicals in the present study were purchased from Sigma–Aldrich Chemical Company (Sigma, St. Louis, MO, USA), unless stated otherwise.

2.2. Establishment and culture of canine dermal fibroblasts

Canine dermal fibroblasts were isolated by previously described methods [14]. Skin samples were obtained from the abdominal region of bitches during routine ovariohysterectomy at Kasetsart Veterinary Teaching Hospital, Kasetsart University, and the veterinary clinic of the Veterinary Public Health Division, Bangkok Metropolitan Administration. They were transported to the laboratory within 2 h in 37 °C phosphate-buffered saline (PBS) supplemented with 2% antibiotics (penicillin and streptomycin). The epidermis and hypodermis were removed with a scalpel blade and the dermis was cut into small pieces (2 mm × 2 mm). Pieces of skin were washed two times with PBS and were placed in culture dishes containing Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Grand Island, NY, USA) supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin). Cells were cultured at 37 °C in a humid atmosphere containing 5% CO_2 and the culture medium was changed every third day. The first outgrowth of dermal fibroblasts was observed after 2–3 d. When cells reached confluence after culturing for 10 d, tissue pieces were removed and cells were washed with PBS and detached from the dish by adding trypsin (0.25% trypsin/EDTA) for 2 min at 37 °C. The cells were then transferred to a centrifuge tube containing 10 mL culture medium and centrifuged at 700 × *g* for 10 min. Cell pellets were resuspended with 1 mL of culture medium, counted with a hemacytometer, and seeded at 5×10^4 cells/mL in 60 mm culture dishes containing DMEM supplemented with 10% FBS and 1% antibiotics. After culture for 5–6 d or after reaching confluence, the cells were trypsinized and frozen with 10% DMSO in culture medium and stored in liquid nitrogen. Frozen dermal fibroblasts were thawed at 37 °C and were cultured in DMEM supplemented with 10% FBS at 37 °C in a humid atmosphere containing 5% CO_2. Cells used for this work were between passages 3 and 4 of culture. To determine doubling time, cells were seeded in culture dishes containing DMEM supplemented with 10% FBS. When confluence was achieved at 5–6 d, cells were trypsinized and counted using a hemocytometer. Cells were then seeded and cultured to reach confluence. Then, the cells were again harvested and counted.

2.3. Flow cytometric cell cycle analysis

Cultured dermal fibroblasts were harvested using 0.25% trypsin/EDTA and resuspended in DMEM at a concentration of 1×10^6 cells/tube. After centrifugation at 700 × *g* for 10 min, the supernatant solution was removed and, while vortexing gently, cells were fixed by drop-wise addition of 0.8 mL cold methanol to the tube containing 0.2 mL of cell suspension. After fixation, cells were stored at −20 °C for 24 h before further analysis. The fixed cells were again centrifuged as above, washed once and re-centrifuged with cold PBS. Cells were resuspended in 0.25 ml PBS containing 0.6 mg/mL RNase and incubated at 37 °C for 30 min. Then, 0.25 mL of propidium iodide solution (PBS containing 50 μg/mL propidium iodide and 0.1% Triton